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THE GOLGI BODIES IN THE NERVE CELLS OF THE CRAYFISH, CAMBARUS

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A survey of the literature on the cytology of the arthropod nerve cell with regard to the Golgi apparatus reveals a controversial heterogeneity of opinion. Poluszynski (1911), who worked with the European crayfish, *Astacus*, reported the presence of the Golgi material in the ganglion cells as discrete elements, quite different from the classical reticular pattern of Camillo Golgi, and was confirmed in this by the findings of Bialkowska and Kulikowska (1912), working on insects. Monti (1915), however, questioned these earlier descriptions, having found in her preparations of the nerve cells of *Astacus* that the Golgi apparatus was present as a fine reticulation, a typical and conventional "apparato interno reticulare" of Golgi. Ross (1922) used as material for his studies the large ganglion cells of the American crayfish, *Cambarus*, but could find neither discrete elements nor the typical reticular apparatus. In spite of this, he accepted unquestionably the structures reported by Monti, describing her preparations as the only ones which showed a Golgi apparatus familiar in structure and appearance, and attributing her success to the superb technique at her command.

The results and conclusions of these earlier investigators were not universally and unqualifiedly accepted, for, to add to the already existing confusion there came conflicting reports from various continental workers. Migliavacca (1930) reinvestigated the nerve cell of *Astacus* and reported a typical Golgi apparatus, taking the view that the discrete elements found by former workers were stages in the development of the chondriosomes. Beams and King (1932) used neither *Cambarus* nor *Astacus*, but drew their conclusions from studies on the nerve cord of insects, that the Golgi substance was present in these cells as discrete bodies distributed through the cytoplasm. Another examination of the

nerve cells of *Astacus* by Dornesco (1934) revealed the Golgi material as discrete, discontinuous elements.

In spite of the diversity of opinion and contradictory reports on this subject, and the failure of Ross to see any Golgi structure in his preparations, there has been no repetition of Ross' work to see whether the Golgi apparatus was demonstrable and what its appearance would be in the nerve cells of *Cambarus*. It was with this purpose in mind that the present work was undertaken.

I wish here to acknowledge my indebtedness to Professor Alden B. Dawson for much interest and invaluable criticism during the course of this work, and to Dr. John H. Welsh, Jr. for identifying the species of crayfish used.

Two species of crayfish were used in this investigation, *Cambarus virilis* and *Cambarus clarkii*. Only healthy active animals were selected from specimens kept in a concrete aquarium tank. In removing ganglia for fixation, portions of the abdominal cord were rapidly dissected out and dropped into the fixative, with no previous anesthesia or killing of the animal; in some cases the thoracic ganglia were removed for study. The time between exposure of the nerve cord and immersion in the fixative never exceeded one minute, the possibility of post-mortem changes in the structure of the nerve cells being thus reduced to a minimum.

The methods used to demonstrate the Golgi apparatus were Ludford's modified Mann-Kopsch technique, the methods of Nassanov-Kolatchev, Cajal, DaFano, and Schridde's process of staining with iron hematoxylin after Champy fixation. The nerve cells proved somewhat refractory to impregnation with osmic acid, the Golgi apparatus not being clearly impregnated until after ten or eleven days of post-osmication at 35° C. in 2 per cent osmic acid. This may explain why Ross was unable to reveal these structures.

The most consistent results were obtained with the Nassanov-Kolatchev technique. Moreover, in several instances, following the routine

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Explanation of Plate I

1. Large ganglion cell from abdominal cord of *C. clarkii*, showing mitochondria and discrete Golgi bodies. Champy-Kull fixation and staining.

2. Large ganglion cell from thoracic cord of *C. virilis*, showing distribution of Golgi bodies. DaFano's cobalt nitrate-silver method.

3. Small ganglion cells from abdominal cord of *C. clarkii*, showing mitochondria and Golgi bodies. Champy-Kull technique.

4. Large ganglion cell from abdominal cord of *C. clarkii*, showing Golgi bodies. Champy fixation followed by Schridde's modified mitochondrial stain.

5. Large ganglion cell from thoracic cord of *C. virilis*, showing Golgi bodies. The osmic impregnation is heavier in the basal region of the cell. Nassanov-Kolatchev technique.

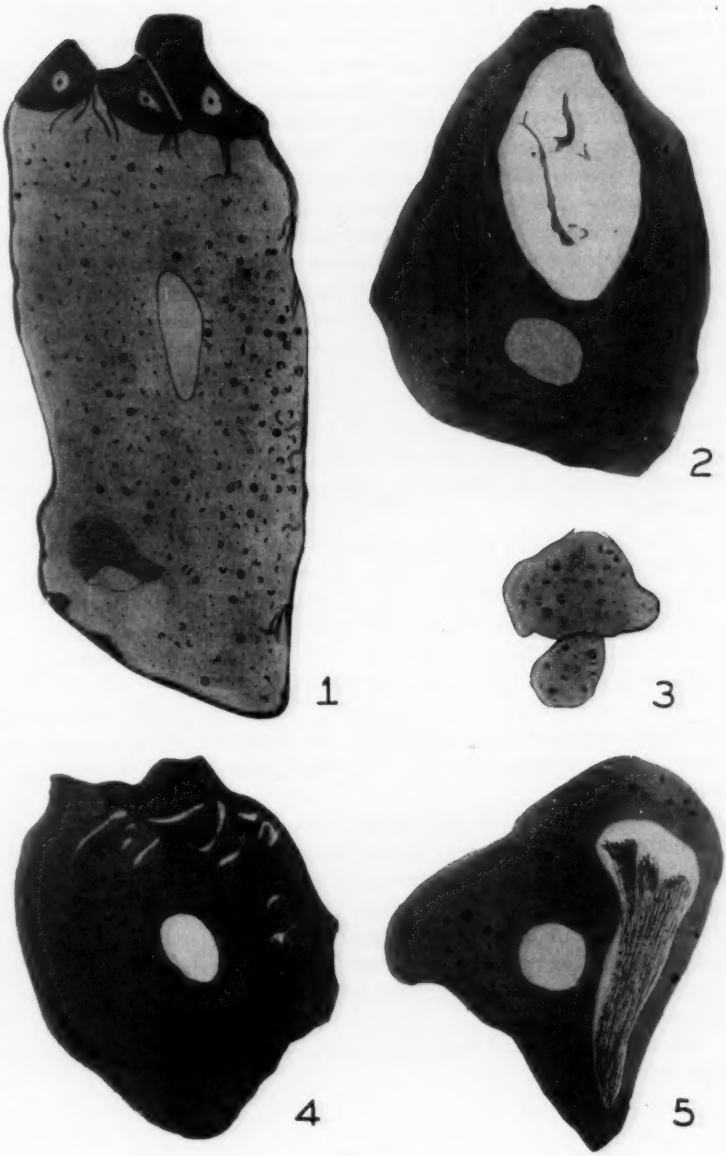

PLATE I

Champy-Kull method for mitochondria, the Golgi bodies were found to have been stained by the acid fuchsin. Fixation in Bouin's picro-formol-acetic acid mixture followed by iron hematoxylin staining gave the negative image of the Golgi structure reported by Beams and King for insect nerve cells. The mitochondria were revealed by fixing and staining according to the method of Champy-Kull.

The degree of impregnation of the osmic acid in the cells varied considerably. In some sections, two cells lying side by side showed differences in the amount of osmication; large cells lying near the periphery of the nerve cord were more heavily impregnated than intermediate and small cells located below the surface; the amount of osmication varied even in the same cell, the peripheral region of the cell being lighter in color than the basal region. Poluszynski attributed this variability to varying physiological states of the cells, and Dornesco attempted to account for a similar phenomenon by explaining it as due to a dilution of the osmic acid by the water content of the cytoplasm. But in the present work, even after renewing the osmic acid daily while the tissues were postosmicating, the same variation in impregnation occurred.

The Golgi bodies are present in the ganglion cells of *Cambarus* as discrete elements. These bodies are plate-like or discoidal in structure with an outer osmiophillic rim and an osmiophobic medulla; in the same cell they are also seen as semi-circles, and curved and straight rods, similar to the structures reported by Poluszynski, Bialkowska and Kulikowska, Beams and King, and Dornesco.

The distribution of the Golgi bodies in the cytoplasm seems to be uniform, with no definite orientation or polarization, though occasional cells were found where they were arranged more or less concentrically about the nucleus.

Nerve ganglia fixed and impregnated by the Cajal and DaFano methods gave good patterns of distribution but inferior morphological pictures in that the Golgi bodies were distorted. Figures 1 and 3 are cells from sections fixed and stained after the Champy-Kull technique. The cells show mitochondria as granules and short rods, and also the Golgi bodies. In no preparation were the mitochondria seen anastomosing to form a reticulum as claimed by Hosselét (1929) for insect nerve cells.

It may be concluded from this paper and from the investigations of others that:

1. There are Golgi bodies present in the ganglion cells of *Cambarus* as discrete elements, now recognized as typical for much invertebrate somatic tissue.
2. They may be readily demonstrated by mitochondrial techniques using acid fuchsin or iron hematoxylin, as well as by the classical osmic acid and silver methods.
3. Ross either did not find the Golgi structures in the nerve cells of *Cambarus* due, possibly, to their refractory behavior to osmic acid, or they were overlooked by him in his preparations.
4. Ross was not justified in accepting the reticular structures reported by Monti for *Astacus* as the typical Golgi apparatus of the crustacean nerve cell.

BIBLIOGRAPHY

BEAMS, H. W., AND R. L. KING, 1932. Cytoplasmic Structures in the Ganglion Cells of Certain Orthoptera. *Jour. Morph.*, **53**: 59.

BIALKOWSKA, W., AND Z. KULIKOWSKA, 1912. Über den feineren Bau der Nervenzellen bei verschieden Insekten. *Bull. Acad. Sci. de Cracov.*, p. 449.

DORNESCO, G. TH., 1934. Recherches sur les constituents cytoplasmiques des cellules nerveuses de Potamobius Astacus L. *Bull. d'Histol. Appliq.*, **11**: 190.

HOSSELÉT, C., 1929. Chondriome à formes d'éléments golgiens dans la cellule nerveuse des Insectes. *Compt. rend. Soc. Biol.*, **100**: 1075.

MIGLIAVACCA, A., 1929. Ricerche sulle strutture citoplasmatiche nelle celle nervose e negli elementi neurilemmali. *Arch. Ital. Anat. d. Embriol.*, **27**: 351.

MONTI, R., 1915. I condriosomi e gli apparato di Golgi. *Arch. Ital. Anat. d. Embriol.*, **14**: 1.

POLUSZYNSKI, G., 1911. Untersuchungen über den Golgi-Kopsch'schen Apparat und einige andere Strukturen in den Ganglienzellen der Crustaceen. *Bull. Acad. Sci. de Cracov*, p. 104.

ROSS, L. S., 1922. Cytology of the Large Nerve Cells of the Crayfish (Cambarus), *Jour. Comp. Neur.*, **34**: 37.

STENOSTOMUM PSEUDOACETABULUM (NOV. SPEC.)

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The species here described fits well into the genus *Stenostomum* as defined by von Graff (1913) but differs sharply from species listed by von Graff (1913) and from those described by Higley (1918), Sonneborn (1930), Kepner and Carter (1931), Nuttycombe (1931), Jones (1932), and Nuttycombe (1932).

Healthy specimens measure from .75 to 2.5 mm. in length. We have never encountered specimens without a fission plane and large individuals frequently show seven well-defined planes (Fig. 1, *F*).

The head is sharply marked off by a constriction just anterior to the mouth and is somewhat spatulate. Posterior to the head the body is cylindrical and tapers gradually into an attenuated caudal region. The posterior end of this region bears a dorsally directed outgrowth (Fig. 1, *A, co*) very similar to that described for *S. rhachiocaudatum*. In *S. pseudoacetabulum*, however, the outgrowth arises at the new fission plane as a postero-dorsal outgrowth from the anterior zoöid (Fig. 1, *D*). In well-fed individuals the body as a whole is very thick and heavy.

In reflected light the animal is white. In transmitted light the integument and anterior region are translucent or pale brownish. The enteric region is gray, brownish, or almost black dependent upon food content. The enteron (Fig. 1, *A, en*) has many glistening vacuoles.

The general ciliary coat is uniform—cilia about as long as epidermis (epidermis other than in free caudal region; here it is thinner than elsewhere) is thick. Non-vibratile cilia, three to four times as long as those in general coat, have been observed in the preoral region but not elsewhere.

Rod-shaped rhabdites (Fig. 1, *E*) vertically disposed, and uniformly distributed lie just beneath the surface of the epidermal cells.

The two ciliated pits (Fig. 1, *A, cp*) are of medium size and laterally placed.

The cephalic ganglia (Fig. 1, *A, cg*) conform to the general pattern for the genus. Light-refracting bodies associated with the ganglia have not been observed.

From above the mouth at rest resembles very strongly a trematode acetabulum. This feature suggested the name *pseudoacetabulum*. This

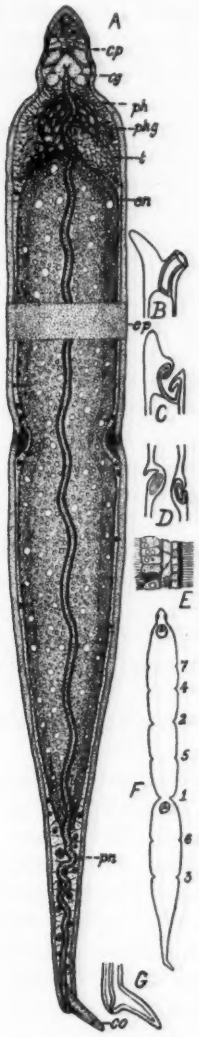

FIG. 1. *A, S. pseudoacetabulum,* dorsal aspect in optical section; *cp,* ciliated pits; *cg,* cerebral ganglia; *ph,* pharynx; *en,* enteron; *phg,* pharyngeal glands; *t,* testis; *pn,* protonephridium; *ep,* dorsal view of epidermis only; *co,* caudal outgrowth. *B,* diagrammatic sketch of anterior region with pharynx everted. *C,* diagrammatic sketch of anterior region with pharynx inverted. *D,* fission plane with developing pharynx and developing caudal outgrowth. *E,* details of body wall and enteron to show endoderm, pseudocoel, and ectoderm. *F,* outline of chain of eight zoöids labelled to indicate relative age of fission planes. *G,* lateral view of caudal outgrowth with terminal protonephridial opening.

sucker-like appearance is due to the fact that the lips are invaginated so deeply as to involve about the anterior one-third of the pharyngeal wall (Fig. 1, *C*). This region may be everted rapidly to form a proboscis-like structure (Fig. 1, *B*).

The pharynx is widened posteriorly to form a cap over the anterior end of enteron. The pharyngeal walls are highly muscular and prominently glandular (Fig. 1, *A*, *phg*). A sphincter occurs between the pharynx and the enteron. The pharyngeal lumen is ciliated. The radial muscle fibers from the pharyngeal wall to the body wall are very numerous.

The enteron (Fig. 1, *A*, *en*) conforms in shape to the body region in which it lies. It terminates considerably anterior to the caudal end of the animal and leaves an enteron-free caudal region. The enteric epithelium is approximately twice as thick as that of the epidermis (Fig. 1, *E*). Its cells are prominently vacuolate and finely ciliated. Occasional granular gland cells occur.

The general mesenchyme is restricted to the pseudocœl and the spaces between the organs. Its cells are of various shapes and sizes.

The protonephridium (Fig. 1, *A*, *pn*) passes posteriorly from the preganglionic region beneath the transverse commissure of the cephalic ganglia, dorsal to the pharynx and enteron and empties terminally beneath the dorsal outgrowth at the posterior end.

About half of the specimens studied were sexually mature as males. The testis (Fig. 1, *A*, *t*) is located above and slightly to the right side of the pharynx.

This species was collected in large numbers from ponds in Bulloch County, Georgia.

Specific diagnosis: Stenostomum pseudoacetabulum differs from all other species of the genus in that its lips, at rest (Fig. 1, *C*), are inverted well into the pharynx. Furthermore, it differs from all other species, except *S. rhachiocaudatum*, in having a single, dorsally-directed, caudal outgrowth. *S. pseudoacetabulum*, however, differs sharply from *S. rhachiocaudatum* in that it has no light-refracting bodies, no vesicular bodies in the epidermis and a terminal opening for the nephridiopore, whereas *S. rhachiocaudatum* has paired light-refracting bodies, prominent vesicular, epidermal bodies and the opening of the nephridiopore is not terminal.

LITERATURE CITED

GRAFF, L. VON, 1913. Turbellaria. II. Rhabdocœlida. Berlin.

HIGLEY, RUTH, 1918. Morphology and Biology of Some Turbellaria from the Mississippi Basin. Biol. Monographs, Univ. of Ill., Urbana, Ill.

JONES, E. RUFFIN, JR., 1932. Stenostomum carnivorum n. sp. *Zool. Anzeig.*, **97**: 292.

KEPNER, W. A., AND J. S. CARTER, 1931. Ten Well-defined New Species of Stenostomum. *Zool. Anzeig.*, **93**: 108.

NUTTYCOMBE, J. W., 1931. Two New Species of Stenostomum from the Southeastern U. S. *Zool. Anzeig.*, **97**: 80.

NUTTYCOMBE, J. W., 1932. Two New Species of Stenostomum. *Zool. Anzeig.*, **101**: 29.

SONNEBORN, T. M., 1930. Genetic Studies on Stenostomum incaudatum (Nov. Spec.). *Jour. Exper. Zool.*, **57**: 57.

THE EFFECT OF CENTRIFUGING ON THE POLARITY OF AN ALGA, GRIFFITHSIA BORNETIANA

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Introduction

The nature of differentiation and particularly the causes of the axial differentiations which define the polarity of an organism, are problems which have been attacked from many different angles. Experimental procedures for a long time centered about the concept of specific organ-forming substances. At first the simple expedient of overturning fragments was utilized to observe the effect of heavier and lighter substances upon regeneration through the action of gravity. This method, employed chiefly with plant material, showed that although orientive adjustments occurred, there was usually little effect upon the actual point of origin of new roots and shoots (e.g. Vöchting, 1885, 1906; Loeb, 1924). Exceptional cases, perhaps most clearly illustrated in the lower plants (Noll, 1900; Wulff, 1910) were explained, often by the authors themselves, as due to the stimulation of preformed centers by accumulated indifferent nutrients.

At Naples, in 1901, Morgan attempted to analyze the effect of gravity on the regeneration of Antennularia through the use of a "rotating wheel." The centrifuge method, initiated by Lyon in 1906 and adopted by many others,[1] may have developed from this simple device. As with gravity, however, it was shown clearly that centrifugal displacement of visible granules is ineffective in altering the normal pattern of growth, except of course in the event of injury. Lillie (1909) therefore concluded that polarity is a property of the ground substance; and Conklin (1931) decided that the effect of extremely high speeds (upon the development of ascidian eggs) is due to the displacement of specific areas in the cytoplasm.

The case which I wish to present in this paper has several points of interest. It is the only one I know of in which a new axis of polarity, involving normal organ formation, is set up by centrifugal force. The

[1] Morgan and Lyon, 1907; McClendon, 1909; F. R. Lillie, 1909; Morgan, 1908, 1909, and 1910; Morgan and Spooner, 1909; Boveri, 1910; Conklin, 1931 and previously.

results are obtained at surprisingly low speeds, which renders improbable the displacement of cytoplasmic areas. The facts are of interest, also, because of the light which they may be construed to throw upon the doctrine of " specific stuffs."

Material and Method

During the course of an investigation on the effect of direct electric current upon regeneration in the alga, *Griffithsia bornetiana* (Schechter, 1934), electrophoresis of chromatophores to the position where rhizoids arose was frequently observed. Preliminary experiments with the centrifuge to determine whether there was any causal relationship between these two phenomena gave negative results, but indicated an interesting effect upon the shoots. The present report is concerned with this effect. The experimental work was done chiefly during the summer of 1934, and the substance of the results presented at the Marine Biological Laboratory at the seminar of August 7, 1934. I wish to acknowledge gratefully helpful conferences with Dr. L. G. Barth.

Freshly collected tufts of the alga were cut into suitably sized fragments and centrifuged continuously for about 24 hours with a force of approximately 150 × gravity. Stratification of the cell contents began in about an hour and after 24 hours the chromatophores were accumulated as a dense cap of material at the centrifugal pole, sharply marked off from the rest of the cell which had become quite transparent. The fragments were then allowed to develop in Syracuse dishes of sea water until new shoots appeared. An ocular micrometer was used for measuring and sketches were made with the aid of a camera lucida.

In the early experiments there was much damage to the material, arising toward the end of the period of centrifuging. By avoiding overcrowding and carrying out the experiments at 19° C. (7–9° below room temperature during the hot weeks of 1934), injury was much reduced and often almost entirely avoided. Under carefully controlled conditions one batch of material was centrifuged continuously for a period of four days with a force of 61 × gravity. Shoots and rhizoids were regenerated.

After the usual amount of centrifuging (about 150 × gravity for 24 hours) it required a week or more before redistribution of the cell contents restored the normal appearance. This unusual duration of stratification may be of significance with respect to the effect upon polarity.

Results

Out of 18 experiments the material died in three, probably due to high temperature, and in 12 of the remaining 15 there was more or less determination of shoot origin in each, as described below:

When the cell apexes were oriented outward from the axis of rotation shoots always appeared in normal positions (Fig. 1, sketches 12, 13, 14). On the other hand, as shown in sketches 1, 2, 6, 8 and Photographs 4 through 12, shoots often arose from the base when the cell

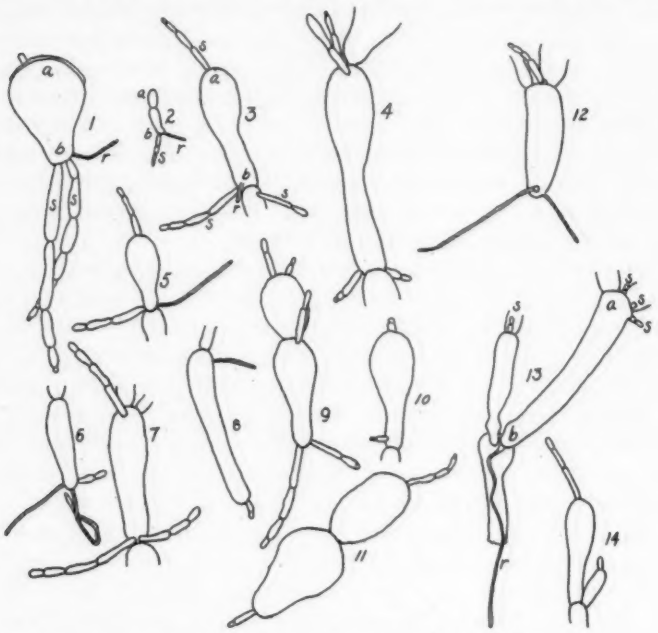
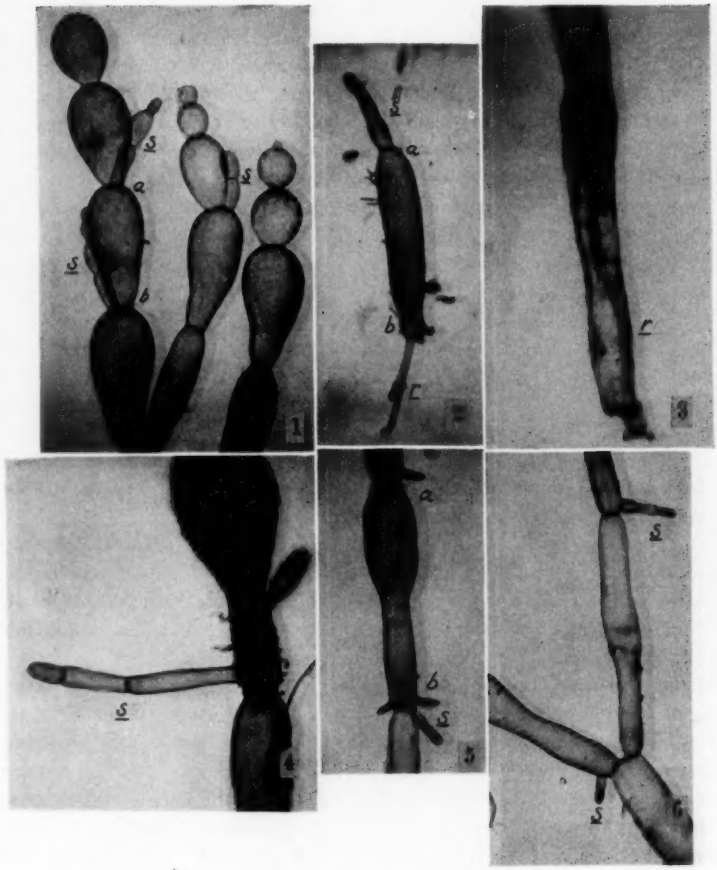


Fig. 1. Camera lucida sketches of experimental material.
Sketches 1–11 of material centrifuged basally. Induced basal, and some shoots in normal position, are shown on the same or different cells.
Sketches 12–14 of material centrifuged apically. The normal polarity was retained. (*a*—cell apex, *b*—base, *s*—new shoot, *r*—rhizoid.)

bases were centrifugally oriented. However, basal shoot formation did not necessarily exclude proliferation from the apexes of the same cells (sketches 3–5, 7, 9–11). It is to be noted that all of the shoots were quite normal in appearance. For comparison several young shoots on control material are shown in Photograph 1. Photographs 2 and 3 are of basally centrifuged cells in which the normal polarity nevertheless remained unaltered.



EXPLANATION OF PLATES

PLATE I

Plates I and II contain photographs of control (Photograph 1) and of experimental material centrifuged basally.

1. Control showing two-, three- and four-celled new shoots in normal apical position.

2. A cell, centrifuged basally, in which the original polarity was retained.

3. The base of a cell with material accumulated during centrifugation. Original polarity was retained in this case as shown by rhizoid in normal position.

4, 5, 6. Baso-lateral shoots following centrifugation. The cells were still part of the original filament, where they occupied a position about midway between apex and base of the plant.

An inspection of the angle of induced shoots upon isolated cells and upon those cells still part of a filament, reveals an interesting situation. The new shoots came off laterally (Photographs 4, 5, 6) when upon cells forming part of a filament. On free cells they usually arose directly from the base (Photographs 8, 10, 11, 12); or there was occasionally a shoot in each position. Photograph 7 shows a male apical cell partially dislodged from the chain with both a lateral and an almost directly basal shoot. In Photograph 9, where the adjoining cell is dead, the basal shoot has grown directly through the dead region. These observations indicate a tendency toward a rather local effect directly in line with the axis of centrifuging.

Unlike shoot formation, rhizoid origin from centrifuged cells was not materially affected. In fact rhizoids often appeared on the cell base together with induced shoots (Photograph 11). In Photograph 12 a new shoot, by forming a rhizoid on its own axis, has completely established independent polarity.

The series of photographs demonstrates also that shoot-forming polarity may be reversed in cells anywhere along the axis of the alga, from the extreme apex to the extreme base.

Data concerned with the frequency with which reversals occur is based mainly upon four experiments. In cells oriented so that heavier materials were thrown toward the apex 229 apical shoots were formed. When oriented in the opposite direction a roughly equivalent number of cells produced 214 apical and 121 basal shoots. The centrifugal forces in these experiments were 100 to 220 $\times$ gravity, an average of 160 $\times$.

It seems interesting that the new shoots were generally smaller in the experiments than in the controls. In one case, for example, 14 typical shoots upon basally centrifuged cells averaged 2.6 cells in number (range 2–5) and 0.75 mm. in length. Where the heavier substances were thrown into the cell apexes 17 shoots, varying from 1 to 6 cells, averaged 3.3 cells and 1.17 mm. in length. Eleven typical shoots upon control material were 3.0 mm. in average length and consisted of from 2 to 9 cells, an average of 6.5. Perhaps correlated with their smaller size is the general observation that new shoots occurred more frequently on centrifuged cells. No exact data on this point are available at present.

These measurements, besides showing a difference between shoots on centrifuged and non-centrifuged cells, also bring out the fact that orientation of the cells during centrifugation affected the size of new shoots. A summation of the data of several experiments indicates that the difference is a general one. One hundred and four apical shoots (unaffected in origin) upon basally centrifuged material averaged 2.9 cells and 0.9 mm. in length; whereas 41 basal shoots consisted of 2.54

cells and were 0.78 mm. long. Also supporting are measurements made in one experiment upon 15 basally centrifuged cells each of which formed both apical and basal shoots, as in sketches 5 and 10, Fig. 1. The average length of the former was 0.85 mm. and of the latter 0.76 mm.

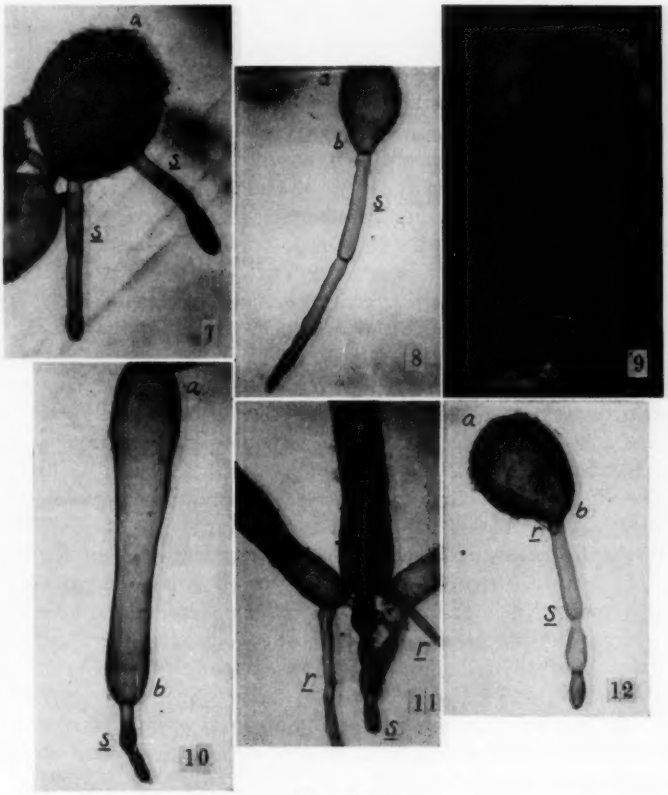


PLATE II

7. An apical cell partially dislodged from the filament. One new shoot formed laterally, one almost directly basally.

8. An isolated apical cell. New shoot directly basal.

9. An apical cell held to the filament by a dead adjoining cell. A basolateral shoot and a basal shoot were formed.

10. A basal cell. Basal shoot on the free basal end.

11. Basal cells. Induced basal shoot and normal basal rhizoids are both present.

12. A basal shoot from an apical cell. The basal cell of the new shoot formed a rhizoid normal to the polarity of the induced shoot.

In all of the instances cited above the basal (induced) shoots were smaller than the apical. If we may assume a fairly regular rate of growth, then the size of shoots is an indication of the time at which they began to form and the difference in size should be a measure of the amount of centrifuging required to induce basal shoot formation.

Discussion

In view of numerous unsuccessful attempts to control polarity by centrifuging and the absence of reports in this direction incidental to the large amount of other work involving the same technique, the results presented in this paper appear to comprise the only case in which a normal determinative effect upon development has been obtained. It is therefore of particular importance to investigate the underlying mechanism of centrifuge action in this material. To be sure, the syncytial nature of the *Griffithsia* cell (see Lewis, 1909) offers a different type of biological system than do animal eggs and embryos, upon which practically all other work involving centrifugation has been done. It may be that some feature of organization peculiar to a syncytium makes the results possible. But in nature *Griffithsia* shoots are formed on the cell apexes regardless of orientation with respect to gravity. We would have to concede to the centrifuge, therefore, a greater efficiency and perhaps a higher specificity in the separation of cellular elements than can be attributed to gravity.

The effect obtained by centrifuging *Griffithsia* also seems, in other respects, somehow unrelated to the action of gravity. Loeb (1894) found that hydranths of Antennularia were regenerated from the upper ends of overturned stems. With plants, where it has been possible to obtain responses other than those of orientation, results have been similar. In all of these cases the new growths formed at the upper ends were those normally found in that position in nature. Quite on the contrary with *Griffithsia;* after centrifuging the new shoots were produced where the heavier materials had been thrown, a position synonymous with the lower end of the organism in the field of gravity; one normally associated with rhizoid formation.

The possibility therefore arises that the effect is due, not to the movement of shoot-forming substances, but to a stimulation set up by an unusual concentration of rather non-specific materials. The simultaneous appearance of shoots on both poles of some of the cells also weighs against the existence of a definite shoot-forming substance, necessarily limited in amount, and is in accord with the above hypothesis. An analogy can perhaps be drawn with activation of unfertilized eggs by various chemical and physical agencies.

May I, in concluding, take this opportunity to acknowledge my indebtedness for help and stimulation to Dr. Charles F. Hunt, whose understanding interest extended from medicine to other fields of science, and whose recent death has removed a rare and valued friend.

Summary

Normal shoots appear upon *Griffithsia* cells at the point where heavier substances are concentrated by prolonged low speed centrifuging. In this way reversal of polarity may be produced anywhere along the plant axis. The possibility is suggested that the centrifuged substances are not directly determinative but act by stimulation.

LITERATURE CITED

Boveri, Th., 1910. Über die Teilung Centrifugierter Eier von Ascaris megalocephala. *Arch. Entw.-mech.*, **30**: 101.

Conklin, E. G., 1931. The Development of Centrifuged Eggs of Ascidians. *Jour. Exper. Zool.*, **60**: 1.

Lewis, I. F., 1909. Life History of Griffithsia bornetiana. *Ann. Bot.*, **23**: 639.

Lillie, F. R., 1909. Polarity and Bilaterality of the Annelid Egg. Experiments with centrifugal force. *Biol. Bull.*, **16**: 54.

Loeb, J., 1894. On Some Facts and Principles of Physiological Morphology. Biological Lectures at the Marine Biological Laboratory in 1893. Ginn and Co., Boston.

Loeb, J., 1924. Regeneration from a Physicochemical Viewpoint. New York.

Lyon, E. P., 1906. Some Results of Centrifugalizing the Eggs of Arbacia. *Am. Jour. Physiol.*, **15**: xxi (Proc. Am. Physiol. Soc.).

McClendon, J. F., 1909. Cytological and Chemical Studies of Centrifuged Frog Eggs. *Arch. Entw.-mech.*, **27**: 247.

Morgan, T. H., 1901. The Factors that Determine Regeneration in Antennularia. *Biol. Bull.*, **2**: 301.

Morgan, T. H., 1908. The Effects of a Centrifugal Force on the Eggs of Cumingia. *Science*, **27**: 446.

Morgan, T. H., 1909. The Effects Produced by Centrifuging Eggs Before and During Development. *Anat. Rec.*, **3**: 155.

Morgan, T. H., 1910. Cytological Studies of Centrifuged Eggs. *Jour. Exper. Zool.*, **9**: 593.

Morgan, T. H., and E. P. Lyon, 1907. The Relation of the Substances of the Egg, Separated by a Strong Centrifugal Force, to the Location of the Embryo. *Arch. Entw.-mech.*, **24**: 147.

Morgan, T. H., and G. B. Spooner, 1909. The Polarity of the Centrifuged Egg. *Arch. Entw.-mech.*, **28**: 104.

Noll, F., 1900. Über die Umkehrungsversuche mit Bryopsis. *Ber. d. Deut. Bot. Gesell.*, **18**: 444.

Schechter, V., 1934. Electrical Control of Rhizoid Formation in the Red Alga, Griffithsia bornetiana. *Jour. Gen. Physiol.*, **18**: 1.

Vöchting, H., 1885. Über die Regeneration der Marchantieen. *Jahrb. f. wissensch. Botanik*, **16**: 367.

Vöchting, H., 1906. Über Regeneration und Polarität bei höheron Pflanzen. *Bot. Zeitung*, **64**: 101.

Wulff, E., 1910. Über Heteromorphose bei Dascycladus clavæformis. *Ber. d. Deut. Bot. Gesell.*, **28**: 264.

CHANGE IN SIZE AND SHAPE OF AGEING EGGS (ARBACIA PUNCTULATA)

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WITH TECHNICAL ASSISTANCE OF VICTOR SCHECHTER AND MILTON LANDOWNE

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Using the method of Lucké and McCutcheon (1926, 1927, 1931), we found that the permeability of unfertilized eggs (*Arbacia punctulata*) increased progressively with age.[1] The factor in their formula that gave us considerable trouble was size of eggs prior to testing. Size may be determined by measuring each egg before as well as during the test. But this is impracticable. Size is usually determined by extrapolation. But several extrapolated values may frequently be obtained, and the resulting permeability rates may be significantly different. Size may be determined by measuring control eggs at successive ages under the same conditions. When eggs were so measured we found that a cyclical change in size occurred with age.

PROCEDURE

Eggs were measured during the summers of 1930, 1931, 1932, and 1933. The *Arbacia* were received directly from the collecting boat. Those females were chosen whose eggs were in good physiological condition, and which had the largest number of spherical eggs. The eggs of each female were strained and washed in 200 cc. sea water, divided into two or more portions, kept in flat finger bowls, at 16.5 to 22.0° C. The temperature at successive ages varied by 1 to 3° C. The supernatant sea water was changed twice daily. Sea water collected at or near high tide was filtered and stored before use. The pH was 8.3.

The diameters of 50 consecutive *spherical* eggs were measured with an ocular micrometer at 660 ×, or with a filar micrometer at 260 ×. Measurements are believed accurate within $\pm 0.4\,\mu$. The probable error is $\pm 0.11\,\mu$.

VARIATION IN SIZE OF EGGS FROM DIFFERENT FEMALES

Table I gives, for 25 females, the number of eggs measured, their age and physiological condition, the average and extreme sizes. One thousand and fifty eggs, in good physiological condition, ¼ to 5 hours

[1] In press.

old, were measured. The average size of eggs from different females varied widely, viz., by 36.9 per cent.

In previous investigations, age and physiological condition have usually not been given, or diminutive (erupted) as well as oversized (dead or fused) eggs have undoubtedly been included in the data

TABLE I

Variation in Size of Freshly-shed Eggs from Different Females

Series	Date	No. of Eggs	Age	Range in Volume	Av. Volume	Cleavage
			hours	$\mu^3 \times 100$	$\mu^3 \times 100$	*per cent*
I_2	7/ 2/32	40	1¾	2262–2514	2431	100
E_2	6/28/32	50	½	2157–2544	2344	—
G_2	7/ 1/32	14	2	2105–2372	2228	—
B	6/30/31	28	½	1957–2276	2128	99
I_3	7/12/33	50	1½	2018–2211	2125	99
F_2	7/18/32	50	5	2029–2236	2120	—
J_3	7/17/33	55	3½	2039–2189	2111	100
$P_{3,3}$	7/31/33	50	4½	1957–2167	2056	100
V	7/29/30	22	¼	1979–2156	2050	—
N	7/14/32	49	4	1962–2103	2049	100
$P_{4,1}$	7/31/33	50	5	1957–2167	2048	100
W	8/ 5/30	40	½	1936–2166	2005	—
G_1	7/21/31	50	1½	1800–2146	1999	93
E_1	6/28/32	50	½	1857–2145	1999	—
$P_{3,2}$	7/31/33	50	5½	1937–2103	1998	100
J_1	8/ 3/31	50	3	1938–2165	1996	99
P_0	7/15/30	9	1½	1858–2139	1979	—
U	7/25/30	12	3	1798–2086	1963	—
F_1	7/18/31	50	1	1761–2082	1959	100
I_1	7/27/31	50	4	1781–1917	1883	100
H	7/11/33	50	3	1762–2060	1909	100
L	7/ 6/33	50	½	1788–1954	1873	99
G_3	7/ 7/33	60	4	1669–1998	1848	100
P_2	7/26/32	30	5	1698–1930	1783	100
K	8/ 6/31	50	3	1724–1897	1776	100
Total 25		1050			Av. 202,640 μ^3	
Variation					36.9%	

(Glaser, 1914*a*). It is also probable that the average size was based on too few eggs (Glaser, 1924; Goldforb, 1917*b*). Lillie (1916) measured 58 eggs, presumably from one female, whose average size was 213,000 μ^3. Lucké, McCutcheon, and Hartline (1931) measured 450 eggs, in good physiological condition, in groups of 50 or 100, from 6 females. The average sizes varied from 182,300 to 224,500 μ^3, mean 201,183 μ^3. E. N. Harvey (1932) quotes another series of measure-

ments by these authors, viz., 550 eggs from 4 females, average size 212,200 μ^3. E. B. Harvey (1932) records an average of 203,690 μ^3 (number of eggs and females not given). In our series, Table I, the averages ranged from 177,600 to 243,100 μ^3, mean size 202,640 μ^3.

The variation in size of eggs from any female is small. The curve of distribution is quite narrow. For the sake of brevity, only the extreme sizes are given in Table I. These extremes varied from 7.2 to 19.7 per cent, average 12.6 per cent. It should be noted that the curve of distribution may be asymmetrical. For example, in Series I only 6 per cent, while in Series G_1, 62 per cent of the eggs were larger than the mode. When the averages were similar, the range may be different. For example, in Series G_1, E_1, and J_1, the average sizes were 199,900, 199,900, and 199,600 μ^3 respectively; the minimal sizes were, however, 180,000, 185,700, and 193,800 μ^3 respectively.

Table I also suggests that size may decrease as the breeding season advances.

The wide variation in size of eggs from different females (Table I) is not due to physiological deterioration, for 99 to 100 per cent cleaved normally, or 95 or more per cent formed parthenogenetic membranes in distilled water; nor is this variation due to age, since eggs of the same age show similar variation in size.

The average of 50 consecutive spherical eggs is not as accurate an index of size as desired. Nevertheless, it is believed that Table I affords a more adequate picture than has heretofore been offered of the large variability in size of freshly-shed eggs of known age and physiological condition from different females, and of the smaller variation in size of eggs from any one female.

It may be noted that eggs from different females differ widely not only in size but in fertilization membrane formation (Goldforb, 1917*a*), cleavage (Goldforb, 1917*b*), and agglutinin (Goldforb, 1929*a*).

Increasing Size During Early Ages

When samples were measured at successive ages there was a progressive *increase* in size during the first 23 to 50 hours.

Series *W*, Table II, illustrates a medium increase. The smallest diameters, between ½ and 24½ hours, increased from 71.67 to 72.53 μ. The largest diameters increased from 74.39 to 75.10 μ. The number of large eggs also increased significantly with age. The average size increased progressively from 200,500 to 207,200 μ^3. The increase during the first 44 hours was 3.07 per cent.

Series *L* illustrates a small increase. The smallest diameters were the same size between ½ and 29½ hours. The maximum diameters

increased slightly, i.e., from 72.0 to 72.6 μ. The number of large eggs increased, between ½ and 8½ hours. The average size increased from 187,300 to 190,800 μ^3, or 1.9 per cent.

Thirteen series are summarized in Table III. The increases in size for the given ages were 1.1, 1.9, 2.1, 2.2, 2.3, 3.0, 3.2, 3.3, 4.7, 5.6, and 5.8 per cent respectively. Table III gives the minimum and maximum

TABLE II

Variation in Size of Eggs with Age

Series	Age	No. of Eggs	Percentage of Eggs							Range in Diameter	Av. Size	Increase or Decrease	Cleavage
			Below 28.0	28.1 to 28.5	28.6 to 29.0	29.1 to 29.5	29.6 to 30.0	30.1 to 30.5	Above 30.5				
			Ocular Units Diameter										
	hours									μ	$\mu^3 \times 100$	*per cent*	*per cent*
W	½	40	0	10	60	17	12	0	0	71.67–74.39	2,005		
	3½	40	0	10	50	27	10	3	0	71.67–74.65	2,018		
	6½	40	0	4	47	35	13	1	0	71.92–74.65	2,025		
	18	40	0	2	45	25	28	0	0	71.92–74.39	2,043		
	24½	40	0	0	55	30	14	1	0	72.53–74.65	2,035		
	30½	40	0	5	57	5	18	3	7	71.67–75.10	2,060		
	44	20	0	0	30	55	15	0	0	71.92–74.91	2,072	+3.0	
	48	20	20	15	55	10	0	0	0	69.43–73.17	1,914	−7.6	
L	½	50	0	46	52	2				69.90–72.00	1,873		99
	3½	50	0	32	56	12				69.90–72.60	1,897		96
	8½	50	0	20	68	12				69.90–72.60	1,908	+1.9	96
	15½	50	0	52	44	4				69.90–72.60	1,890		89
	29½	50	0	50	44	6				69.90–72.60	1,866		
	37½	50	20	64	16	0				68.70–72.00	1,800		
	47½	50	6	64	30	0				69.20–71.91	1,832	−4.2	73

diameters at the initial age and at the age when eggs were largest. It will be noted that the minimum diameters increased with age in most series; the maximum in all but one series. The abbreviated table does not, however, show the progressive increase in numbers of large eggs. Table III shows an increase of 2,400 μ^3 to 11,500 μ^3. For reasons to be given later, the increase with age is believed to be even larger.

It is therefore concluded that, under the stated conditions, a significant increase in size of control eggs occurred as the eggs aged; that the increase may be measurable by the third hour, may continue for 22 to 50 hours, and may be as much as 5.8 per cent.

Decreasing Size During Late Ages

In every series in which eggs were measured at late and frequent ages there was a much greater change in size but in the reverse direction. In Series *W* (Table II) the minimum diameter decreased from

Table III

Summary of Thirteen Series Showing Cyclical Change in Size with Age

Series	Ages Measured	Increase		Late Decrease	Diameter			
					Initial Age		Intermediate Age	
					Minimum	Maximum	Minimum	Maximum
	hours	μ^3	*per cent*	*per cent*	μ	μ	μ	μ
P_0	1, 2, 3, 20, *23*,* 27, 30, 43, 54	11,500	5.8	4.6	70.80	74.21	71.82	75.22
U	3, 5, 7, 9, *24*,* 27, 30	9,200	4.7	†	70.20	73.58	70.71	75.40
V	⅒, 2, 5, 8, 21, 28, *34**	11,500	5.6	9.7	72.21	74.29	72.21	77.90
W	½, 3, 6, 18, 24, 30, *44*,* 48	6,200	3.0	7.6	71.67	74.39	71.92	74.91
B	½, 2, 5, 10, *23*,* 28, 32, 48, 52, 56, 72	2,400	1.1	9.5	72.04	75.76	73.28	76.01
F_1	1, 8, 24, 31, *47*,* 55	4,300	2.2	0.4	69.43	73.40	69.92	74.39
G_1	1, 8, 24, 31, *50**	6,300	3.2	—	69.93	73.90	71.92	74.39
I_1	4, 42	0	0	1.8	69.80	71.53	69.45	72.15
J_1	3, *31**	4,500	2.3	†	71.67	74.39	71.92	74.63
K	3, *25**	3,700	2.1	†	68.94	71.18	69.43	71.68
I_2	2, 6	600	0.2	†	75.60	78.30	76.50	78.60
L	½, 3, *9*,* 15, 29, 37, 47	3,500	1.9	4.2	69.90	72.00	69.90	72.60
P_2	5, *22**	5,800	3.3	†	68.70	71.70	69.00	71.70

* Indicates ages when maximal size was observed.
† Eggs not measured at late ages.

71.92 to 69.43 μ. The maximum diameter decreased from 75.10 to 73.17 μ. The curve of distribution shifted sharply towards the left. The average size decreased 7.6 per cent. In Series *L* there was a slower decrease of 4.2 per cent.

Table III shows a decrease during late ages of 4.6 per cent (Series *Po*), 9.5 per cent (Series *B*), and 9.7 per cent (Series V).

Comments on Cyclical Change in Size

With age, eggs enlarged and the curve of distribution shifted to the right. At intermediate ages some eggs continued to swell, while others, having reached their maximum size, progressively diminished in size. The curve became bimodal. The condensed tables do not adequately show this phenomenon. At later ages all the eggs have

shrunken and a monomodal curve is reëstablished, but is now shifted sharply to the left.

Eggs swelled more than indicated in the tables. Since the initial size was measured $\frac{1}{10}$ to 5 hours after shedding, the later the initial age, the greater had been the swelling. Hence the increase based upon this late initial size is correspondingly too small. It is also improbable that the eggs were measured exactly when they reached their maximum size. If measured before or after this maximum, the increase would be correspondingly too small.

At late ages, eggs tend to fuse into giant eggs (Goldforb, 1913). Upon death, intact eggs swell considerably. Many eggs cytolyze with closely adhering fragments. All these eggs were excluded. The increased size discussed above refers to a change in living eggs, which were able to cleave and were morphologically indistinguishable, except for color and granulation, from freshly-shed eggs.

Increase in size with age may not be attributed to greater flattening against the bottom of the dish. McCutcheon, Lucké and Hartline (1931) have demonstrated that flattening does not occur in freshly-shed *Arbacia* eggs. We believe that flattening did not occur during the first ca. 25 hours. During late ages, however, flattening probably did take place, for the viscosity of the egg progressively decreased and the egg membrane softened considerably. Yet in spite of this probable flattening and pseudo-enlargement, during late ages, the eggs progressively decreased in size.

The change in size may not be attributed to changes in H ion concentration of the medium. The supernatant sea water was progressively more acid. The pH changed from 8.3 to 7.9. But this degree of change is too small (Lucké, McCutcheon, 1926), and does not account for the cyclical change in size.

As shown elsewhere,[2] permeability to water increased progressively and markedly with ageing of eggs. It is this increased water intake which we believe to be one of the major factors in the increasing size of ageing eggs.

The decreasing size during late ages may be accounted for on the basis of the following observations. Beginning about 23 hours after shedding, the plasma membrane ruptured and a small pellicle, about $1\frac{1}{2}$ μ diameter, was ejected. The pellicle, containing minute grey granules and fluid, rounded out quickly and immediately formed a membrane. The ruptured membrane closed completely in 1 to $1\frac{1}{2}$ minutes. Within 2 minutes the egg, except for adhering pellicle, appeared normal and when fertilized cleaved normally.

[2] In press.

At later ages (about 30 to 40 hours) a larger cone formed. The membrane burst at or near the apex of the cone. The pellicles were larger (i.e. to 3.75 μ diameter) and contained small and large granules. The egg membrane reformed more slowly (2 to 3 minutes). The egg near the erupted area remained pale for several minutes. Later (50 or more hours) more eggs ruptured, the pellicles were larger and formed in succession. At this time pellicles graded into the larger globules characteristic of cytolyzed eggs.

The smallest pellicles, not including water discharged into the medium, were estimated as ca. 1 μ^3. With age, their size increased to and beyond 27.5 μ^3. If the discharged water be included, the mass of ejected materials is correspondingly greater. It is this repeated ejection of substances that is held responsible in largest part for the decreasing size of the eggs during late ages.

TABLE IV

Showing Variation in Percentage of Ellipsoid Eggs, Freshly-Shed, 16 Females

Series.....	B_1	L_1	E_1	E_3	E_2	G_9	G_1	B_3	L_2	I	J	K	N	F	P	B_3
Ellipsoid eggs, %	62	42	26	32	45	10+	50	40	20	35	37	15	18	29	35	36
No. of eggs examined.....	74	50	68	72	74	50	28	—	50	62	50	50	49	70	46	50
Age in hours....	½	½	½	½	½	2	2	2	2	2	3	3	4	5	5	5
Cleavage (per cent).....	99	97	*	*	*	93	*	*	99	100	99	100	100	*	100	*

* In distilled water formed 95 per cent or more parthenogenetic membranes.

Not only does size change cyclically but the rate of fertilization, of cleavage, and of sperm agglutination (Goldforb, 1918, 1929*a* and *b*) also show a similar cyclical phenomenon, with age.

It has been suggested that in calculating the permeability rate, the size of eggs at all ages be averaged; but from the above discussion it is evident that any constant size is contrary to the facts, and since a small error in size may considerably alter the permeability rate, a much more accurate rate may be obtained only when the cyclical trend and the size of eggs at each age are known.

CHANGE IN SHAPE OF AGEING EGGS

Variation in Percentage of Globular Eggs from Different Females

The percentage of globular eggs in different females varied widely. Table IV summarizes the results in 16 series. Eggs from 5 females examined within ½ hour after shedding contained 62, 45, 42, 32 and

26 per cent ellipsoid eggs respectively. In 5 females, whose eggs were examined 2 hours after shedding, the percentages were 50, 40, 35, 20 and 10 + per cent respectively. When 3 hours old, there were 37 and 15 per cent ellipsoid eggs. When examined 5 hours after shedding, the percentages were 36, 35, and 29 per cent respectively.

These percentages are not strictly comparable. Age is one variable factor. In Table V, for example, the percentage of ellipsoid eggs decreased between ½ and 5 hours from 62 to 26 per cent. A second variable factor is the method of determining ellipsoid eggs. In Series *B* the two extreme diameters were measured in each egg. In other series

TABLE V

This table illustrates the large percentage of ellipsoid eggs at the initial age, the degree of ellipsoidality, and the increased percentage of globular eggs with age. Eggs from 1 female aged at 19 ± 0.3° C. Series *B*1.

Difference in diameters (μ)	Age (*hours*). ½	2½	5	10½	23	28	32	48	52	56	72
Globular Eggs (per cent)											
0– .3	38	60	64	90	98	100	100	100	100	96	96
Non-globular Eggs (per cent)											
.4– .6	16	8	2	4	0	0	0	0	0	0	0
.7– 1.3	10	14	6	2	0	0	0	0	0	0	0
1.4– 2.5	14	12	6	0	0	0	0	0	0	4	2
2.7– 3.7	6	4	10	2	0	0	0	0	0	0	0
3.8– 5.0	8	0	6	4	0	0	0	0	0	0	0
5.1– 7.5	2	0	2	0	0	0	0	0	0	0	0
7.6–10.0	2	0	2	0	0	0	0	0	0	0	0
10.1–12.5	4	0	0	0	1	0	0	0	0	0	0
12.6–15.0	0	2	2	0	0	0	0	0	0	0	2
No. Eggs Measured	74	50	50	51	50	50	50	50	50	40	40
Cleavage (*per cent*)	99	—	—	96	91	—	90	60	—	12	6

these diameters were measured in eggs which could not definitely be classified by inspection. In other series, classification was by inspection only. Ellipsoid eggs may also appear circular in optical plane. Finally females were chosen (except Series *B*) with the least number of ellipsoid eggs.

Hence it may be concluded that ellipsoid eggs occurred in all females studied; that the percentage ranged from 10 + to 62; that the actual percentages are probably greater. Since cleavage was normal and ranged from 97 to 100 per cent [3] (except Series G_1), neither ellipsoid shape *per se* nor the increased numbers with ellipsoid shapes are criteria of physiological deterioration.

[3] The eggs of the other females were also in good physiological condition, for over 95 per cent formed parthenogenic membranes in distilled water.

Increasing Globular Shape with Age

Series *B* (Table V) illustrates the increasing globular shape with age. The eggs of one female were measured at different ages between ½ and 72 hours after shedding. The two extreme diameters were measured in consecutive eggs, 40 to 74 at each age. When ½ hour old, only 38 per cent were globular, i.e., the extreme diameters at right angles to each other differed by 0 to 0.35 μ. Of the 62 per cent ellipsoid eggs, 16 per cent had diameters differing by 0.4 to 0.6 μ, 10 per cent differed by 0.7 to 1.3 μ, 14 per cent by 1.4 to 2.5 μ, etc. The most ellipsoid eggs had diameters differing by 15.0 μ.

At successive ages between 2½ and 28 hours, the percentage of globular eggs increased progressively, viz., from 38 to 60, 64, 90, 98, and 100 per cent respectively. All eggs remained spherical between 28 and 52 hours.

Though only 38 per cent of the freshly-shed eggs were spherical, yet they were in good physiological condition; 99 per cent cleaved normally. During the next 10 hours, the percentage of spherical eggs increased from 38 to 90 per cent. During this time there was little or no deterioration. Cleavage was normal and decreased from 99 to 96 per cent, probably due to delayed rather than reduced cleavage.

Between 28 and 52 hours, there was no change in shape, yet deterioration was very rapid. Cleavage was increasingly irregular and fell from 91 to 34 per cent. Between 56 and 72 hours, with extreme deterioration 4 per cent ellipsoid eggs reappeared.

Seven other series gave essentially similar results.

Factors Involved in Change of Shape

Unripe eggs increase in size and are spherical before maturation. It is generally assumed that eggs are normally shed soon after maturation, but eggs may be retained within the body for long periods and be deteriorated when shed. This is indicated by the following: *Arbacia* were kept in separate large aquaria, the water changed twice daily. When shed the eggs were immediately inseminated. Such eggs frequently gave low percentage cleavage. Extreme deterioration was also observed in eggs from freshly-opened females, collected after severe storms or extreme heat, also towards the close of the breeding season.

It is believed that any condition that delays shedding of eggs results in increasing the number and size of ripe ova. As a consequence there is an increasing mechanical pressure of eggs against one another; and it is this pressure that gives rise to increasing ellipsoid shape. The greater the duration and degree of pressure, the greater the number of ellipsoid

eggs and the greater the degree of ellipsoidality. This mechanical pressure exerts little or no injurious effect. Injury appears to be due to ageing processes within the body of the female. Upon shedding, the mechanical pressure is released, and increasing permeability to water further aids in the return to globular shape.

Conclusions

Cyclical Change in Size

The average size of freshly-shed eggs, in good physiological condition, varied widely in the 25 females studied, viz., 177,600 to 243,100 μ^3, or 36.9 per cent.

The eggs from any one female varied within much narrower limits, viz., 13,600 to 38,700 μ^3, or 12.6 per cent.

Size of eggs increased with age in all 13 series.

The increase may be evident by the third hour and end between 23 and 50 hours.

The increase ranged from 1.1 to 5.8 per cent. These percentages exclude fused and dead eggs.

The minimum and maximum, the mode and average, as well as the number of large eggs, all increased progressively with age.

During late ages, size decreased progressively by 4.2 to 9.7 per cent. This excluded fragmented eggs.

Size increase was accompanied by little deterioration; size decrease by considerable deterioration.

Increasing size is attributed to increased water intake; decreasing size to ejection of pellicles and water.

The cyclical change in size of control eggs must be known in the determination of the permeability rate.

Change in Shape

Ellipsoid eggs occurred in all 16 females. They ranged from 10 + to 62 per cent. The degree of ellipsoidality is given in text.

These females were selected for least ellipsoid eggs.

Ellipsoid eggs were in excellent physiological condition.

With age, eggs became spherical. All eggs were spherical in 8 to 37 hours, and remained spherical thereafter.

Shape is not correlated with physiological condition.

Ellipsoid shape is attributed to retention of mature eggs within the body, and therefore to increased numbers and size. The result is mechanical pressure and ellipsoidality. Shedding releases this pressure. Increased water intake aids in rounding out and enlargement of eggs.

BIBLIOGRAPHY

GLASER, O. C., 1914*a*. *Biol. Bull.*, **26**: 84.
GLASER, O. C., 1914*b*. *Biol. Bull.*, **26**: 367.
GLASER, O. C., 1924. *Biol. Bull.*, **47**: 274.
GOLDFORB, A. J., 1913. *Biol. Bull.*, **24**: 73.
GOLDFORB, A. J., 1917*a*. *Carnegie Inst. Publ.*, No. 251, p. 73.
GOLDFORB, A. J., 1917*b*. *Proc. Nat. Acad. Sci.*, **3**: 241.
GOLDFORB, A. J., 1918*a*. *Biol. Bull.*, **34**: 372.
GOLDFORB, A. J., 1918*b*. *Biol. Bull.*, **35**: 1.
GOLDFORB, A. J., 1929*a*. *Biol. Bull.*, **57**: 333.
GOLDFORB, A. J., 1929*b*. *Biol. Bull.*, **57**: 350.
GOLDFORB, A. J., 1929*c*. *Biol. Bull.*, **57**: 389.
HARVEY, E. B., 1932. *Biol. Bull.*, **62**: 155.
HARVEY, E. N., 1932. *Biol. Bull.*, **62**: 141.
LILLIE, R. S., 1916. *Jour. Exper. Zool.*, **21**: 369.
LUCKÉ, B., AND M. MCCUTCHEON, 1926. *Jour. Gen. Physiol.*, **9**: 709.
LUCKÉ, B., H. K. HARTLINE, AND M. MCCUTCHEON, 1931. *Jour. Gen. Physiol.*, **14**: 405.
MCCUTCHEON, M., AND B. LUCKÉ, 1926. *Jour. Gen. Physiol.*, **9**: 697.
MCCUTCHEON, M., AND B. LUCKÉ, 1927. *Jour. Gen. Physiol.*, **10**: 659.

VISCOSITY CHANGES IN AGEING UNFERTILIZED EGGS OF ARBACIA PUNCTULATA

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In previous studies it was demonstrated that ageing unfertilized eggs of *Arbacia punctulata* undergo a series of changes including rate of fertilization, membrane formation, and cleavage (Goldforb, 1918*a* and *b*), agglutinin concentration (Goldforb, 1929*a* and *b*), and size (Goldforb, in press). Permeability to water and stretching and bursting of the plasma membrane in hypotonic sea water changed markedly with age. These studies will be reported later. The present study is devoted to a consideration of the viscosity changes.

PROCEDURE

Eggs from each female, if in good physiological condition, were washed in 200 cc. sea water. Aliquot portions were transferred to flat finger bowls with 150 cc. sea water. Morning and evening the supernatant water was removed, the eggs transferred to clean bowls, and sea water added. Sea water was collected in quantity at high tide, filtered and stored. The pH was 8.3. The temperature in the bowls varied from 18 to 22° C., but during an experiment within 2.5°, usually within 1.5° C. At successive ages eggs from the same bowl were centrifuged by a slight modification of the method developed by Heilbrunn (1928).

CONSTANT CENTRIFUGAL FORCE, VARYING TIME, EARLY EXPERIMENTS

In 1930,[1] eggs were centrifuged in 2 mm.-bore tubes in a small electric centrifuge. Immediately after centrifuging, the eggs were examined in sea water. After preliminary trials a period of centrifugation was chosen that gave the following zonation:

(1) Oil cap sharply defined.

(2) Hyaline zone in most eggs extended 45 to 55° [2] from center of oil cap.

(3) Twenty consecutive eggs, with planes of zoning at right angles to the horizontal, were measured.

[1] Dr. V. Schechter assisted in these experiments.

[2] I.e., degrees on circumference from center of oil cap.

(4) Hyaline zone near oil cap free of granules, near grey zone containing numerous scattered granules.

(5) Grey and red zones sharply differentiated.

At successive ages, eggs were centrifuged at the same speed, but the time was varied to approximate the same degree of zonation. Increase in time denoted relative increase in viscosity, and vice versa. The results may be summarized as follows:

Between 10 minutes and 3 hours after shedding, viscosity increased in most experiments. The exceptions will be discussed later.

Between ca. 3 and 30 hours, *viscosity progressively increased with age* in every experiment. For example, in one experiment the time of centrifuging required to approximate the same degree of zonation increased progressively from ¾ to 2½ minutes.

Beginning about 40 hours after shedding, *viscosity progressively decreased.*

This cyclical change in viscosity occurred in all experiments. That the changes were significant was shown by the following: Repetitive tests agreed within 15 seconds; centrifuging time increased with age by 105 or more seconds. Though the temperature during some tests rose to 3½° C., yet when those tests were chosen in which the temperature did not vary beyond 0.5°, the same changes occurred. Though the number of eggs examined at each age was small, yet all experiments gave similar results.

Constant Force and Time, Change in Percentage of Zoned Eggs

In 1931, with the same centrifuge and tubes, the centrifugal force and time were constant at successive ages. The centrifuge registered 4,444 r.p.m. (without load). As the result of preliminary trials a period of centrifugation of 2 minutes duration was usually chosen. This time included $7\frac{1}{2} \pm \frac{1}{2}$ seconds to attain maximal speed, but did not include 26 ± 2 seconds for the centrifuge to stop. The voltage was constant during the day. Tests were made when no other large electrical apparatus was used on the same line. During a test the temperature in the centrifuge head varied between 21.4° and 22.4° C., usually within 0.6°. Immediately after centrifuging, the eggs were transferred to 0.4 per cent formalin (Howard, 1931) and examined within 2 hours. About 100 consecutive eggs, lying at a proper angle, were examined at each age and classified as follows:

Group 1	*Group 2*	*Group 3*
Oil cap very distinct	Same	Less distinct
Hyaline zone, granules few	Many	Very numerous
Hyaline zone, 60–70°	45–60°	Less than 40°
Hyaline grey border, thin straight line	Very ragged	Loose band
Hyaline and grey zones sharply different in color	Less sharp	Intergrades
Oil to grey zone, ca. 15 units	Ca. 12	Less than 10

A typical experiment is summarized in Fig. 1. The eggs of one female were centrifuged for one minute. The number of eggs recorded at each age varied from 84 to 247 (average 194). Between 2¾ and 22 hours after shedding, the adequately zoned (Group 1) eggs *decreased* progressively from 51 to 14 per cent. This decrease denoted correspondingly greater viscosity. After 22 hours, however, there was a reversal. The percentage of Group 1 eggs *increased* from 14 to 51 and then to 84 per cent. This reversal or liquefaction occurred during rapid deterioration.

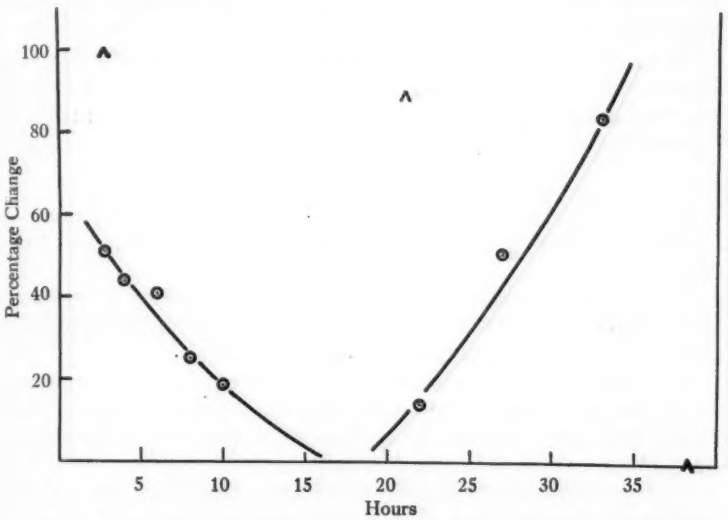

FIG. 1. Centrifuged at 4,444 r.p.m. for 1 minute at 21.5° C. The percentage of eggs zoned to a fixed standard decreased progressively during the first ca. 20 hours and increased thereafter. A decrease in percentage indicates corresponding increase in viscosity and vice versa. ⊙ = per cent zoned; ∧ = per cent cleavage of control eggs.

Similar results were obtained in a second experiment. Eggs 1½ hours old, centrifuged for 2 minutes, gave 85 per cent which were

sharply zoned. Between 1½ and 29 hours the percentage of eggs of Group 1 decreased progressively from 85 to 0 per cent, while the percentage of eggs of Group 2 increased from 5 to 94 per cent. When 48 hours old, Group 2 eggs had decreased from 98 to 38 per cent, while Group 3 (the least zoned) eggs had increased from 6 to 62 per cent. In the case of the older eggs, in order to avoid crushing them at the bottom of the tubes, second samples were centrifuged for one minute only. Between 24 and 48 hours the eggs of Group 2 decreased progressively from 91 to 24 per cent, while Group 3 eggs increased from 9 to 76 per cent.

In a third experiment, the percentage of adequately zoned (Group 1) eggs decreased, between 5 and 48 hours, from 100 to 71 per cent. When centrifuged during late ages, for 1 minute only, the percentage of Group 2 eggs decreased from 85 to 17 per cent.

In the last two experiments liquefaction appears to have begun about the forty-eighth hour. At this age the zonation of many eggs was greater and the hyaline zone contained fewer granules and was much deeper than at any previous age.

It was therefore concluded that viscosity was progressively increased during early and intermediate ages; that there were indications of a progressive liquefaction during late ages; that the degree of change is indicated by the change in percentage of eggs zoned to a fixed standard.

Same Force, Varying Time, Improved Technique

In 1932, viscosity was determined by the following improved technique:

1. *Temperature.*—The centrifuge head was cooled by a regulated flow of ice water. The temperature was recorded at the beginning and end of each test. It varied within 1°, usually within 0.5° C.

2. *Centrifugal Force.*—A powerful Emerson hand centrifuge was used. The time necessary to attain maximum speed was reduced from 7½, in preceding experiments, to 1 second. The time necessary for stopping was reduced from 26 to 1 second.

3. *Low Centrifugal Force.*—After repeated trials a relatively low centrifugal force was selected, viz., 1,750 gravities.

4. The standard of zonation approximated that of Group 2 in previous experiments. At this low centrifugal force and short duration, a small change in centrifuging time gave rise to readily detectable changes in zonation. Hence accuracy of tests was increased.

5. Two or more determinations were made at each age, one usually for the same time as in the accepted previous test, the other for longer

or shorter time. *The objective was to produce not only the same zonation, but the same percentage of similarly zoned eggs.*

6. *The Avoidance of Injury at Bottom of Tube.*—To prevent smashing of eggs at the bottom of the tube, at late ages, a capillary drop of eggs was added to a fixed level of isosmotic solution of C.P. cane sugar solution. The eggs were not injured by the sugar solution, for they cleaved like the control eggs. Nor was their viscosity altered by the solution.

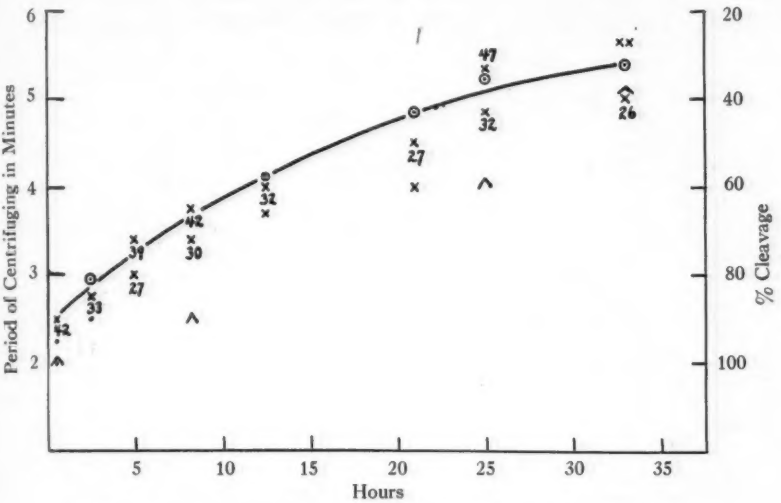

FIG. 2. Centrifuged at 1,750 gravities at 21.5° C. for indicated minutes to approximate same percentage of similarly zoned eggs. Centrifuge time increased during the first 33 hours.

× = observed time in minutes.
⊙ = calculated time.
· = insufficiently zoned.
×× = overzoned.
∧ = percentage cleavage.
Numbers = percentage of eggs zoned to standard.

7. *Sensitivity of Test.* An increase of 10 to 15 seconds longer centrifuging definitely increased the percentage of zoned eggs. For example, in one experiment, eggs centrifuged for 2 minutes resulted in no eggs zoned to standard. With each increase of 10 seconds centrifugation, the percentage of zoned eggs increased to 18, 53, and 86 per cent respectively. In another experiment centrifugation for 1¾ min-

utes produced 34 per cent zoned eggs. Fifteen seconds more centrifugation gave 52 per cent. Repetitive tests varied 0 to 8 per cent.

Cyclical Viscosity, Eggs Centrifuged in Sea Water

In Experiment T_1 (Fig. 2), the first test was made 17 minutes after shedding. When centrifuged for 2 and 2¼ minutes, the eggs were insufficiently zoned. When centrifuged for 2½ minutes, 42 per cent (of 165 eggs) were zoned to a carefully noted standard.

When 2½ hours old, one sample was tested for 2½ minutes as before. The eggs were not sufficiently zoned. The second sample, centri-

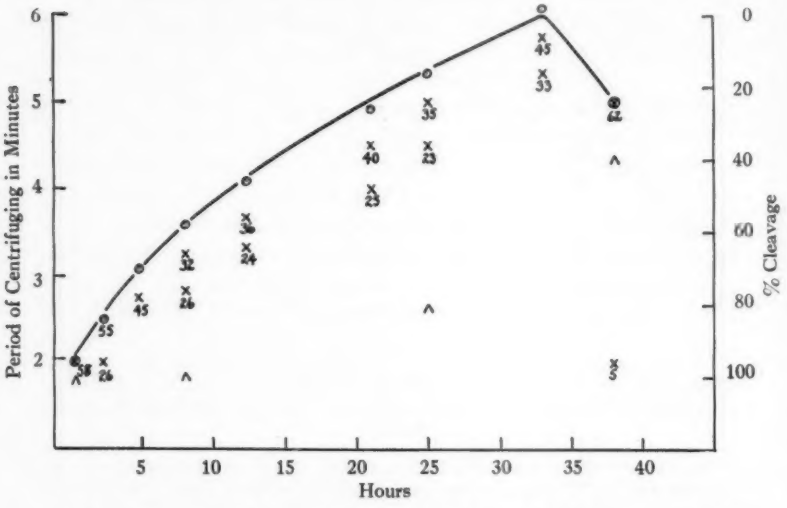

Fig. 3. Same conditions and symbols as in Fig. 2. Shows *increased* viscosity during first 33 hours and decreased viscosity thereafter.

fuged 30 seconds longer, contained only 33 per cent zoned to standard. The estimated time necessary to produce the same percentage of eggs zoned to the same standard as in the initial test was 2′ 55″.

When 5 hours old, 2 samples were again centrifuged, one for 3 minutes (to approximate the previous estimated 2′ 55″) and another for 3′ 25″. The first contained only 27 per cent, the second 39 per cent zoned eggs. The latter approximates the initial 42 per cent. Hence 55 seconds longer centrifuging was required at this age to produce the same zonation as in the ¼-hour old eggs.

Similar double tests were made at successive ages to 33 hours. The

time required to approximate the same percentage of equally zoned eggs increased with age as follows: 2.5, 2.7, 3.4, 3.7, 4.0, 4.5, 5.3, and 5.5 minutes respectively. The estimated time increased progressively from 2½ to 5⁷⁄₁₂ minutes (Fig. 2). Viscosity increased 116 per cent.

The experiment was repeated with similar results (Fig. 3). Several facts deserve brief mention.

(1) For similar ages the increase in centrifugal time was greater, i.e., from 2 to 6½ minutes, an increase of 202 per cent.

(2) Liquefaction occurred after 33 hours. Between 33 and 38 hours the centrifugal time decreased from 6½ to 5 minutes. Though

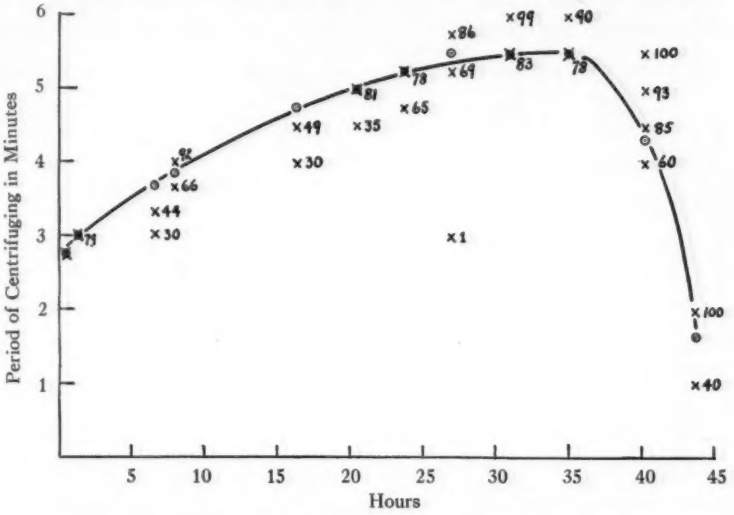

FIG. 4. Centrifuged in isosmotic sugar solution 1,460 gravities. Same symbols as in Figs. 2 and 3. Shows increased viscosity to thirty-fifth hour and progressive decrease to forty-fourth hour.

the eggs were less viscous than at the thirty-third hour, they were much more viscous than at the initial age, for when centrifuged for 2 minutes (the initial time) only 5 instead of 58 per cent of the eggs zoned to standard.

(3) Liquefaction occurred when the eggs were markedly deteriorated.

CYCLICAL VISCOSITY, EGGS CENTRIFUGED IN ISOSMOTIC SOLUTION

When centrifuged in an isosmotic C.P. cane sugar solution (density 1.085), the eggs were scattered below the sugar level, where the force was 1,460 ± 100 gravities.

In Experiment T_3, for example, 2 to 6 tests were made at each age. An average of 173 eggs were examined after each test. Between 1½ and 35 hours the time of centrifuging, required to produce the same percentage of equally zoned eggs, increased progressively from 2¾ to 5½ minutes (Fig. 4).

When eggs were 27 hours old, it was necessary to centrifuge for 5½ minutes to zone 79 per cent (the initial per cent) to standard. When centrifuged for 3 minutes (the initial duration), only 1 per cent were zoned to standard. This affords another measure of the increased viscosity at this age.

After 35 hours there was rapid liquefaction. When 40½ hours old, a sample centrifuged for 5½ minutes gave 100 per cent either

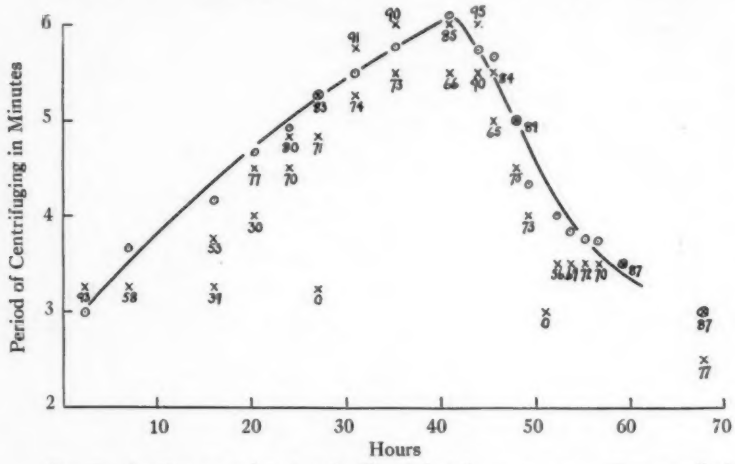

FIG. 5. Another experiment as in Fig. 4, but for more ages. Same cyclical viscosity with age.

zoned to standard or overzoned. Less centrifugation, i.e., 4⅓ minutes, sufficed. When 43½ hours old, eggs were centrifuged for 4½, 4, 3½, 3, and 2 minutes respectively. All these gave 100 per cent zoned or overzoned eggs. The time required to zone to standard had decreased from 5½ to 1⅔ minutes.

In Experiment T_4, Fig. 5, tests were made as in the previous experiment but over a longer series of ages. Viscosity increased progressively during the first 40 hours. The increase was from 3 to 6½ minutes or 100 per cent. Between 44 and 68 hours viscosity decreased rapidly. At late ages, even at the reduced time of centrifugation, nearly all the eggs were overzoned, so that liquefaction was greater than indicated by the figures.

It is therefore concluded that *ageing eggs undergo a cyclical change in viscosity, increasing during the first ca. 35 hours and decreasing thereafter,* and that the increase is of the order of 2 to 3 times the initial viscosity. The subsequent decrease is even greater.

Viscosity of Ripe vs. Unripe Eggs

In the preceding experiments there were a few unripe eggs in nearly every test. None of these, however, showed any trace of zonation. To determine the relative viscosity of ripe and of unripe eggs, females were chosen that contained the largest number of unripe eggs,

Table I

Viscosity of Unripe Eggs

Exp. No.	♀ No.	Age	Centrifugation		Medium	Zoning Unripe Eggs	Zoning Ripe Eggs
			Force	Time			
		hours	*gravities*	*minutes*			
1	1	2	1750	1⅚	Sea Water	0	68% zoned to standard
	2	4	1750	2	" "	0	67 " " "
2	1	1	710	7½	Isosmotic Sugar Sol.	0	30 " " 90°
	1	2	710	10	" " "	0	50 " " "
	1	3	710	15	" " "	0	100 " " 110°
	1	4	710	25	" " "	0	100 " " 120°
3	1	2⅙	1750	1¾	" " "	0	Most " " 45°
	1	1⅚	1750	2	" " "	0	" " " 55°
	1	1¾	4000	1½	" " "	0	" " " 65°
	1	1⅔	4000	2½	" " "	0	" " " 75°
	1	1½	7000	3	" " "	0	" " " 90°
	1	1⅙	7000	5	" " "	0	" " " 110°

and were subjected to longer durations and greater centrifugal forces. Table I summarizes 3 experiments.

Experiment I

Eggs were centrifuged in sea water at 1,750 gravities for 2 minutes. Sixty-eight and 67 per cent of 400 ripe eggs were zoned to standard. None of the unripe eggs in a sample of 1,600 eggs showed any evidence of zonation.

Experiment II

Eggs were centrifuged at 710 gravities for 7½ to 25 minutes. When centrifuged for 25 minutes, all ripe eggs were much overzoned; the hyaline grey border extended from 90 to 120°; yet none of the unripe eggs showed any trace of zonation. A few small unripe eggs formed pseudopodial processes.

Experiment III

Eggs were centrifuged at increasing forces, viz., 1,750, 4,000 and 7,000 gravities for 1¾ to 5 minutes. With each increase in time or force the hyaline zone deepened and the number of scattered granules therein decreased. At the greatest centrifugal force all ripe eggs were not only overzoned but elongated considerably, as described in detail by E. B. Harvey (1932). ***Not a single unripe egg was elongated, and none showed any sign of zonation.***

It is therefore concluded that *either the gravimetrically separable substances are not organized in the unripe egg or being present the viscosity is more than eleven times greater than in ripe eggs.*

Unripe eggs vary in size to a very much greater degree than ripe eggs, yet no difference in viscosity was detectable between the very small (young), and the large (older) unripe eggs. These observations suggest that, with maturation, there is not only a very profound but possibly a very rapid liquefaction. Liquefaction appears to be far greater than during polar body formation or cleavage, or following changes of temperature, salts, etc. (Heilbrunn, 1921, 1924, 1928).

Discussion

It was shown above that in the life history of the *Arbacia* egg there were five viscosity phases, viz.:

1. *Extreme Viscosity, Unripe Eggs.*—Whatever their age (or size) all unripe eggs were extremely viscous. None could be zoned by the considerable forces and durations used. That unripe eggs were more viscous than ripe ones was noted by Paspaleff (1927) and by Heilbrunn (1928). Our study corroborates these findings and supplies a quantitative measure of the difference, namely that unripe eggs are at least 11 times more viscous than freshly-shed ripe eggs.

2. *Liquefaction.*—Upon maturation there was a very marked and probably very rapid liquefaction in all eggs.

3. *Increasing Viscosity, Ripe eggs, Early Ages.*—All ripe eggs progressively increased in viscosity with age. The rate of increase was slower when the eggs were aged within the body, faster when they were aged in sea water. Viscosity increased during the first ca. 35 (22 to 41) hours after shedding. The increase was 2 to 3-fold.

4. *Decreasing Viscosity, Ripe Eggs, Late Ages.*—After ca. 35 hours there was a reversal, namely, a progressive liquefaction. The rate was faster and the total change was greater than during earlier ages.

5. *Extreme Viscosity, Death.* Our evidence is not conclusive whether at the greatest ages death was directly accompanied by coagula-

tion or whether extreme liquefaction occurred first, followed quickly by coagulation.

These five phases are diagrammatically represented in Fig. 6.

Extent of Change.—These viscosity changes are greater than those which occur under a variety of other conditions, but comparisons are not readily made, either because the changes have not been given quantitatively or because different units have been used. In Table II such a comparison is attempted. This table shows that viscosity in-

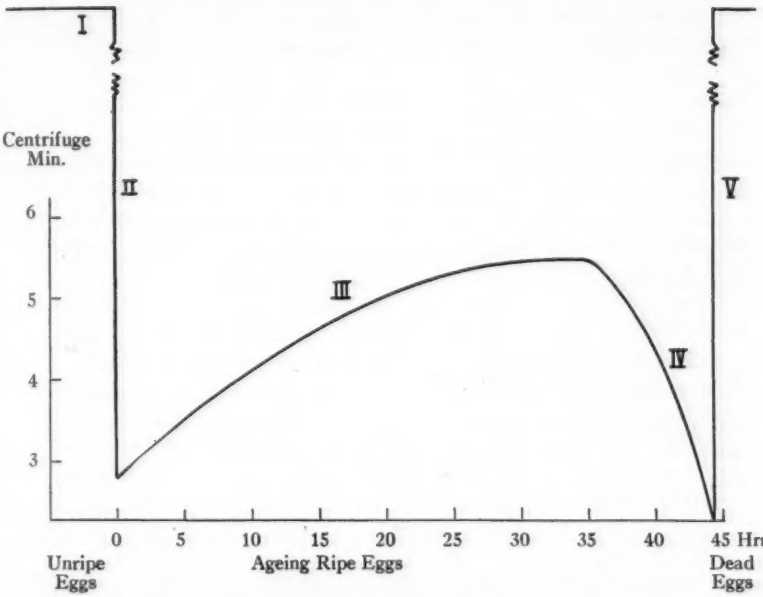

FIG. 6. Diagrammatic representation of the five phases in viscosity of the egg, viz.

I. All unripe eggs at any age, extremely viscous.
II. Maturation, extreme and rapid liquefaction.
III. Ripe eggs, early and intermediate ages, progressively increased viscosity.
IV. Ripe eggs, late ages, much deterioration, progressive liquefaction.
V. Dead eggs, coagulation.

creased during mitosis of *Arbacia* eggs by two-thirds. Larger changes occurred with temperature, HCl, NH_4OH, ultraviolet light, etc., viz., 2¼ to 4 x. The largest change heretofore described occurred during mitosis of *Cumingia eggs,* viz., 5 to 8x. During ageing of *Arbacia* eggs, there were both positive and negative changes of 2 to 11x.

The factors which play a minimal or no rôle in these changes include body fluid, temperature, and pH.

Modification of Egg by Body Fluid.—Body fluid decreases cleavage (Lillie, F. R., 1923; Ephrussi, 1925). Bŏguchi (1930) believed that this was due to intestinal, not to body fluid. Body fluid alters the reaction of eggs to calcium (Heilbrunn, 1925, 1928). In a later study it will be shown that body fluid does decrease cleavage, gives rise to irregular cleavage, accelerates swelling in hypotonic solutions, and increases viscosity. In these experiments body (perivisceral) fluid was withdrawn by pipette through the oral disc, centrifuged, and the supernatant fluid used. The freshly-shed "dry" eggs or eggs from removed

TABLE II

Comparison of Viscosity Changes under Various Conditions

Author	Inducing Conditions	Cell	Viscosity Change
Heilbrunn, 1920	Mitosis	Arbacia Eggs	+ ⅔
Pantin, 1924	Temperature −0.7 to 30° C.	Nereis "	− 3⅗
Heilbrunn, 1921	" 2 to 15° C.	Cumingia "	+ 2¼
" "	" 15 to 30° C.	" "	− 2¼
Barth, 1929	CO_2, pH 8.3 to 5.0	Arbacia "	+ 2½
	HCl " 8.3 to 3.0	" "	+ 2½
	NH_4OH " 8.1 to 11.0	" "	+ 4
Heilbrunn and Young, 1930	Ultra-violet ray	" "	+ 3 to 4
Heilbrunn, 1921	Mitosis	Cumingia "	+ 5 to 8
Goldforb	Unripe eggs	Arbacia "	+11
	Ripe eggs, early ages	" "	+ 2 to 3
	" " , late "	" "	− 3 to 4
	Dead "	" "	+11

ovaries were immediately transferred to this fluid. At successive ages one sample of eggs washed in sea water and another unwashed sample were centrifuged simultaneously in sea water. The body fluid eggs were in every experiment more viscous than eggs from which the body fluid had been washed away. The difference was small but definite. By ca. the sixth hour, viscosity was the same in both kinds of eggs. Therefore neither the marked liquefaction upon maturation, nor the striking increase in viscosity with age may be attributed to the effect of body fluid. It was pointed out above that in a few of the early experiments there was no rise in viscosity during the first 5 hours. This we believe was due to a comparison of eggs whose body fluid had not been thoroughly washed away (hence with greater viscosity) with eggs at the next age when washing had been complete.

Temperature and Viscosity.—In *Nereis* eggs a rise in temperature from — 0.7 to + 30° C. decreased viscosity 3½ times (Pantin, 1924). In *Cumingia* eggs a similar increase in temperature gave rise to a cyclical change in viscosity with maxima at 0, 15, and 32° C. (Heilbrunn, 1924). In our experiments there were five phases with maximal changes of 11 times, but the variation in temperature was within 2.25° C. When those tests were selected during which the temperature had not changed more than 1 or 0.5° C., the same viscosity changes occurred. Temperature, therefore, did not give rise to the marked viscosity changes in ageing eggs.

Hydrogen Ion Concentration.—Acid tends to increase, alkali to decrease viscosity (Jacobs, 1922; Barth, 1929); but viscosity begins to change in acid medium below pH 7.8 (Barth, 1929). In our experiments the transfer from body fluid to sea water decreased viscosity slightly. This was probably due to the change from the acid body fluid, viz., pH 7.4, to alkali sea water, viz., pH 8.3. With ageing, the pH of the supernatent sea water decreased slowly but not below 7.9. Hence this change in hydrogen ion concentration *per se* is not a significant factor in the large viscosity changes during ageing. During latest ages, liquefaction may possibly be due in part to the rapid and considerable inflow of sea water rendering the interior less acid.

Granule Size.—Increased viscosity may be due to the formation of new granules or the increased size of old granules (Heilbrunn, 1928). We were unable, as were previous workers, to demonstrate such changes. Conversely the decreased viscosity during latest ages may be due to diminution of the granules in one or more layers. The four zones [3] were present at all ages, but their volumes changed during latest ages. The oil zone appeared to be unchanged. The hyaline zone, however, was much larger, in extreme instances two or more times the size at previous ages, when the same forces and durations were used. This zone was entirely free of grey granules. More significant is the diminution of both grey and red zones. They shrank from a previous ca. 120° [4] to about ⅓ or 45° in depth. This may mean either a diminution in size of the same number of granules or a loss in number of granules or both. Either supposition may account for the liquefaction at these late ages, but due to this change in depth of zones, an exact comparison of the viscosity at these late ages with the viscosity at earlier ages may not therefore be made.

Ion Penetration.—Rate of ion penetration may change with age. Osterhout (1926) and Brooks (1929) found that K accumulated in the

[3] The fifth zone, described by E. B. Harvey (1932), was not studied.
[4] Measured in degrees on the periphery.

cell sap of ageing *Valonia*. Stiles and Kidd (1919), studying the relative penetration of K, Ca, Na, and Mg in ageing carrot and potato cells, discovered that calcium changed most with age. The effect of changing concentration of ions with age requires further study. The calcium change is now under investigation.

Permeability.—Viscosity and water intake both increased during mitosis and cell division (Heilbrunn, 1920*a*; Haberlandt, 1919, 1920). In our next study it will be shown that permeability to water progressively and markedly increased in ageing *Arbacia* eggs. Increased viscosity in these eggs occurred with increasing permeability to water. Both increased during the first ca. 35 hours after shedding. Thereafter permeability increased much further, while viscosity was reversed. Similar reversal of viscosity occurred with increasing CO_2 (Jacobs, 1922), increasing alkalinity (Barth, 1929), increasing temperature (Heilbrunn, 1920*b*, 1924). It is extremely interesting that Dhar (1930) obtained a reversal of viscosity in ageing hydrophyllic colloids. In our experiments the liquefaction at late ages may be due in part to excess water, to decreased acidity of interior of egg, or to excess calcium.

Injury.—Without attempting to define injury, it may be said that increasing permeability and viscosity occurred with little or no protoplasmic deterioration. Deterioration was measured by decreased and by irregular cleavage. In Experiment T_2, Fig. 3, for example, between ¼ and 8 hours, the viscosity increased 79 per cent, i.e., the centrifugal time necessary to zone to standard increased from 120 to 215 seconds. During these ages cleavage was normal and decreased but 1 per cent (i.e., from 100 to 99). Between 8 and 33 hours viscosity increased to 204 per cent. Irregular cleavage began at the twenty-first hour. Cleavage decreased at the thirty-third hour to 80 per cent. Viscosity, during these ages, increased with progressive injury. Buenning (1926) had observed this phase only. During later ages there was considerable and rapid deterioration. This was associated with decreased viscosity.

Summary

1. Relative viscosity was determined by (*a*) change in percentage of eggs zoned to a fixed standard, at constant centrifugal force and time; (*b*) change in time at a given force to produce same percentage of equally zoned eggs.

2. Ageing unfertilized eggs manifested five major phases in viscosity, viz.

(*a*) Unripe eggs. No trace of zoning could be induced. They were at least eleven times more viscous than mature eggs.

(*b*) All mature eggs were readily zoned at much lower forces and duration. Maturation is accompanied by profound and apparently rapid liquefaction.

(*c*) During the first ca. 35 hours after shedding, ripe eggs progressively *increased* in viscosity, 2 to 3 ×. At constant force and time, the zoned eggs decreased with age from 85 to 0 per cent. To produce the same percentage of equally zoned eggs, the centrifuging time increased from ca. 2 to 5 minutes. Similar results were obtained when eggs were centrifuged in isosmotic sugar solution.

(*d*) With further ageing, viscosity was reversed. There was progressive liquefaction. The decrease was 3 to 4 ×.

(*e*) Upon death viscosity was again sharply increased at least eleven times.

3. The amount of change with age is greater than that during mitosis, or induced by heat, acid, light, etc.

4. Temperature and acid are excluded as factors in the cyclical viscosity changes with age.

5. Perivisceral fluid is also not responsible.

6. Increasing permeability to water is associated with increasing viscosity during early and intermediate ages. During late ages, with excess water intake, viscosity is reversed.

7. Viscosity increased during early ages with no detectable injury. It increased further with progressive injury, i.e., during intermediate ages. It decreased during the period of most rapid injury.

BIBLIOGRAPHY

BARTH, L. G., 1929. *Protoplasma,* **7**: 505.
BOGUCHI, M., 1930. *Protoplasma,* **11**: 432.
BUENNING, E., 1926. *Bot. Arch.,* **14**: 138.
BROOKS, S. C., 1929. *Protoplasma,* **8**: 389.
Dhar, N. R., 1930. *Jour. Phys. Chem.,* **34**: 549.
EPHRUSSI, B., 1925. *C. R. Acad. Sci.,* **180**: 775.
GOLDFORB, A. J., 1918*a*. *Biol. Bull.,* **34**: 372.
GOLDFORB, A. J., 1918*b*. *Biol. Bull.,* **35**: 1.
GOLDFORB, A. J., 1929*a*. *Biol. Bull.,* **57**: 333.
GOLDFORB, A. J., 1929*b*. *Biol. Bull.,* **57**: 350.
GOLDFORB, A. J., in press.
HABERLANDT, G., 1919. *Sitzungsber. d. k. Preus. Akad. d. Wiss.,* Berlin, pp. 322 and 721; 1920, p. 323.
HARVEY, E. B., 1932. *Biol. Bull.,* **62**: 155.
HEILBRUNN, L. V., 1920*a*. *Jour. Exper. Zoöl.,* **30**: 211.
HEILBRUNN, L. V., 1920*b*. *Biol. Bull.,* **39**: 307.
HEILBRUNN, L. V., 1921. *Jour. Exper. Zoöl.,* **34**: 417.
HEILBRUNN, L. V., 1924. *Am. Jour. Physiol.,* **68**: 645.
HEILBRUNN, L. V., 1925. *Science,* **61**: 236.
HEILBRUNN, L. V., 1927. *Arch. Exp. Zellf.,* **4**: 246.
HEILBRUNN, L. V., 1928. The Colloid Chemistry of Protoplasm, Gebruder Borntraeger, Berlin.

HEILBRUNN, L. V., AND R. A. YOUNG, 1930. *Physiol. Zool.*, **3**: 330.
HOWARD, E., 1931. *Biol. Bull.*, **60**: 132.
JACOBS, M. H., 1922. *Biol. Bull.*, **42**: 14.
LILLIE, F. R., 1923. Problems of Fertilization, Chicago, p. 172.
LILLIE, R. S., 1909. *Am. Jour. Physiol.*, **24**: 14.
LILLIE, R. S., 1911. *Am. Jour. Physiol.*, **28**: 197.
LILLIE, R. S., 1912. *Am. Jour. Physiol.*, **29**: 372.
LILLIE, R. S., 1913. *Am. Jour. Physiol.*, **31**: 255.
OSTERHOUT, W. J. V., 1926. *Proc. Soc. Exper. Biol. and Med.*, **24**: 234.
PANTIN, C. F. A., 1924. *Jour. Marine Biol. Ass.*, **13**: 331.
PASPALEFF, G., 1927. *Publ. d. Stazione Zool. d. Napoli*, **8**: 1.
STILES, W., AND F. KIDD, 1919. *Proc. Royal Soc.*, B., **90**: 487.

THE PHYSIOLOGY OF DIGESTION OF PLANKTON CRUSTACEA

I. Some Digestive Enzymes of Daphnia

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This paper constitutes the first report of a biochemical assay of the digestive enzymes of *Daphnia.*

The food of plankton Crustacea has been a contestable subject since Pütter (1909) postulated that most aquatic organisms derived much of their nutrition from dissolved organic matter. The experiments contrary to this theory culminated in the work of Krogh (1930), who found dissolved organic substances of no importance in the nutrition of aquatic animals and of Stuart, et al (1931), who raised bacteriologically sterile *Moina* and found them unable to subsist on dissolved nutritives —particulate food alone could furnish a supporting diet. "Staubfeine" detritus appeared to Naumann (1918) to play the most important rôle in the nutrition of cladocerans. He also (1921) determined a renewal coefficient of the intestinal contents (15–30 minutes) in several species of the same group which demonstrated the short period that food remains in their digestive tracts. Klugh (1927) found some Entomostraca able to utilize fine detritus but showed their chief food to consist of planktonic Chlorophyceae. Many workers point to the importance of bacteria as food. It is not unreasonable to suppose that they make up a part of the filterable food of plankton Crustacea, for Juday (1934) calculates them to be 1 per cent of the dry organic matter found in the centrifuge plankton of Trout Lake.

Birge and Juday (1922 and 1934), in a chemical study of particulate and dissolved organic matter in southeastern and 529 northeastern Wisconsin lakes, find considerable available food stuffs. In the latter group of lakes the organic matter in the centrifugable plankton consisted of 37 per cent crude protein, 4 per cent ether extract and 59 per cent carbohydrate. In the former report, analyses of algae show them to have a higher protein content than carbohydrate.

The experiments reported in this paper show that a digestive mechanism exists in *Daphnia* capable of utilizing protein, carbohydrate, and fat.

Acknowledgment is appreciatively given to Professor C. Juday for

suggesting the problem, to Professor H. C. Bradley, Department of Physiological Chemistry, under whose direction this work was carried out and who furnished laboratory facilities; also to Dr. H. D. Baernstein for instruction in the use of physico-chemical apparatus.

Materials and Methods

Daphnia magna was cultured in large battery jars and butter tubs, on a medium of sheep manure and acid phosphate. Frequent seining yielded sufficient *Daphnia* for limited analysis only. Pure cultures of *D. pulex* were netted from Lake Monona in the spring and fall of the year. Large quantities were obtained from this source.

Finely ground casein, on which the indicators neutral red and brom-cresol-phenol had been adsorbed, was fed to *D. magna*. The color change of the indicator was observed in progress through the alimentary tract and the pH approximated. The pH was found to vary from 6.8 in the anterior end of the tract to 7.2 at the caudal end. This result indicated a tryptic type of proteolytic digestion. Rankin (1929) found a range of 6.8–8.0 in *Simocephalus*.

In the preparation of the extract, *D. pulex* were strained from the water, dehydrated and partially defatted by treatment with acetone. Defatting was continued with petroleum ether. One gram of residue was powdered in an agate mortar and extracted for twenty-four hours with 100 cc. of 50 per cent glycerol. The filtered extract contained proteinases, carbohydrate and fat-digesting enzymes. Fresh hog pancreas with a strip of duodenum were treated in the same manner as the *Daphnia*. This extract was compared with *Daphnia* extract in some of the experiments. For micro-analysis 50 intestines were dissected from *D. magna* and extracted with 50 per cent glycerol.

Examination of the Extract

The Proteinases

In order to micro-analytically detect the presence of a proteinase in *D. magna*, the Gates (1927) photographic plate method was modified to show qualitatively the proteolytic activity of the glycerol extract of *D. magna* intestines. Drops of this extract definitely digested the gelatin film of a photographic plate showing the presence of a proteinase in the digestive tract of *Daphnia*.

For quantitative analysis of the *Daphnia* proteinase, the viscosity method of Northrop (1922) was modified for use. With the aid of the Ostwald viscosimeter, it was possible to follow the hydrolysis of gelatin by a proteinase. The activity of the enzyme is measured in terms of

decreasing viscosity (ΔV) of the substrate. A special gelatin from Swift and Co. was used for the first experiments. Solutions of 3 per cent concentration were made up in a phosphate buffer at pH 7.4, preserved in thymol and kept in test tubes (5 cc./tube) at 3° C. Test tubes of gelatin were placed in a water bath of 34° C.; the temperature was kept constant by a heating unit, agitator, and toluene regulator. When the gelatin reached bath temperature, it was transferred to Ostwald viscosimeters. To these were added 0.5 cc. of the 1 per cent glycerol extract. Viscosity readings were taken as often as possible for the first 15 minutes. After that, readings were made at 10-minute

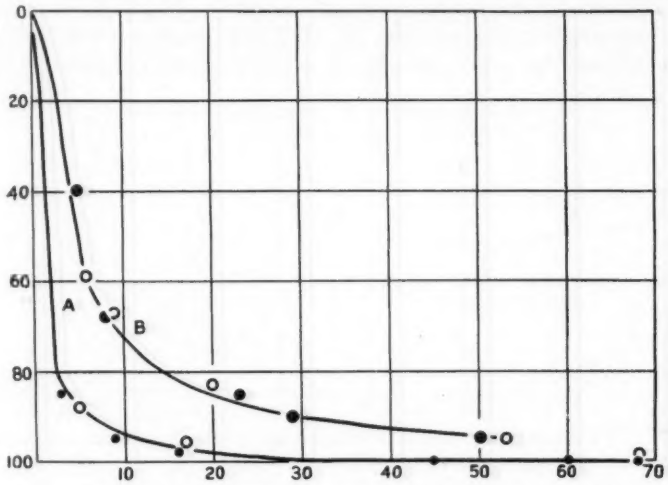

FIG. 1. Hydrolysis of gelatin at pH 7.4 by proteinases of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents decreasing viscosity (ΔV); the abscissa is the time in minutes.

intervals for one hour. To the control tubes was added 0.5 cc. of 50 per cent glycerol. The gelatin-digesting ability of 1 per cent glycerol extracts of both *D. pulex* and hog pancreas were determined and compared. Fig. 1 shows the hydrolysis of gelatin by the proteinases in the extracts of both *Daphnia* and hog pancreas. The curves represent the mean of two runs against a control and were duplicated twice. They are typical hydrolysis curves, falling suddenly at the onset of cleavage. The curves then level off, but *A* gradually approaches *B*. The 1 per cent extract of *Daphnia* decreased $84\Delta V$ in 20 minutes while a 1 per cent extract of hog pancreas fell to $100\Delta V$ in the same time.

Effect of pH on Enzyme Activity

The character of an enzyme is determined by the pH of optimum activity. Mammalian peptic enzymes are most active at pH 1.0, tryptic enzymes pH 7–8 and katheptic enzymes pH 4–5. The subsequent procedure was followed in determining the effect of pH upon proteinase activity of the *Daphnia* extracts. The 1 per cent extract used in the above experiment was diluted to contain 1.5 *Daphnia* proteinase units. A unit was defined as the amount of enzyme necessary to cause a decrease of 20ΔV of 1.5 per cent Sargent's gelatin in 20 minutes; pH 7.4, temperature 34° C.

Test tubes of 1.5 per cent Sargent's gelatin were buffered in a series at hydrogen-ion concentrations of 1–10 and preserved with thymol. The pH was determined with the aid of the quinhydrone electrode. To

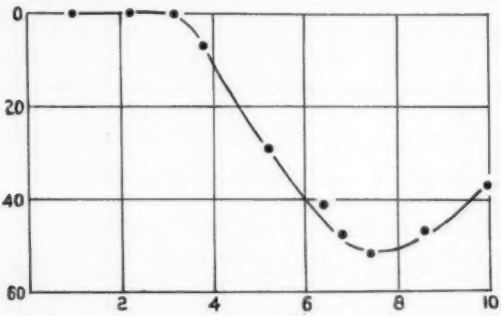

Fig. 2. Activity of *Daphnia* proteinases—effect of pH on gelatin hydrolysis. Decreasing viscosity (ΔV) is the ordinate; the abscissa represents pH.

5 cc. of gelatin at a desired pH, 0.5 cc. of enzyme solution containing 1.5 units was added. Viscosity readings were made and a graph constructed for proteinase activity. The readings at the end of 50 minutes, for each of the pH 1–10 runs, were taken and plotted against pH. The activity of the proteinase at any pH can be read from Fig. 2. The optimum activity was reached at pH 7.4. Its activity decreased on the alkaline side of pH 7.4. On the acid side, the enzyme was found to be inactive at pH 3.2. The graph was constructed from the mean of two runs at each pH. A control was used in all cases and the experiment duplicated three times.

Amylase

The amount of maltose produced by the action of 1 per cent extract on a 3 per cent corn starch solution was iodometrically titrated by the

Baker and Hulton (1920) method for the estimation of sugars and used as an indication of amylase activity. A 10-cc. sample starch solution, pH 7.4, was diluted to 50 cc. and used as the substrate. To this was added 1 cc. of extract and thymol for preservative, and it was then kept at 37° C. Every 15 minutes 5-cc. samples were withdrawn and titrated. The increase in the amount of thiosulphate used in the titration was equivalent to the same amount of maltose liberated in the digest. Duplicate samples were used. The curves in Fig. 3 represent the mean of two runs. The control showed no hydrolysis during the run.

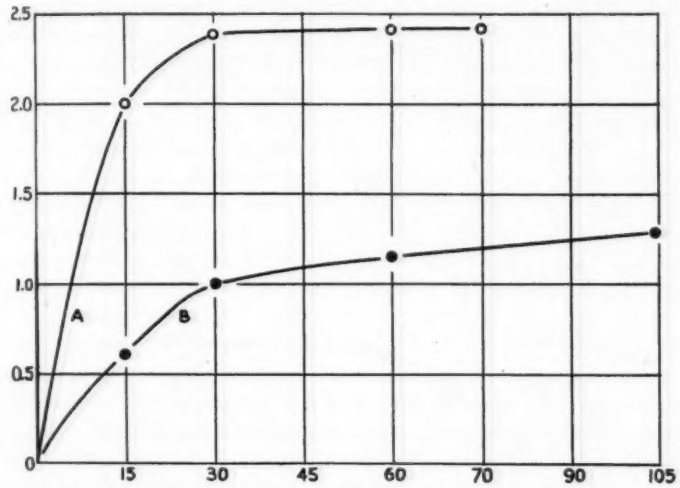

FIG. 3. Hydrolysis of starch at pH 7.4 by amylase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 maltose; the abscissa is the time in minutes.

The concentration of amylase is much greater in the glycerol extract of hog pancreas than in the *Daphnia*. After 30 minutes the hydrolysis of the starch by 1 per cent hog pancreas extract was equivalent to 2.4 cc. of N/20 maltose, while in the same time 1.0 cc. was the total amount produced by the *Daphnia* extract.

LIPASE

For the determination of lipase activity, 50 cc. of 4 per cent tributerine were emulsified with sodium glycocholate. To this were added 2 cc. of 1 per cent extract. The digest was placed at 37° C. and 10-cc. samples were withdrawn hourly and titrated with N/20 NaOH. The

increase in acidity due to the liberation of fatty acids by the hydrolytic action of the lipase in the *Daphnia* extract and hog pancreas extract was recorded in terms of N/20 NaOH. The results are shown in Fig. 4. The experiments were duplicated and run with controls. The presence of a lipase in the *Daphnia* extract is clearly demonstrated. The mean acidity at the end of four hours was equivalent to 0.9 cc. of N/20 NaOH in the case of 1 per cent glycerol extract of *Daphnia* and 2.6 for hog pancreas.

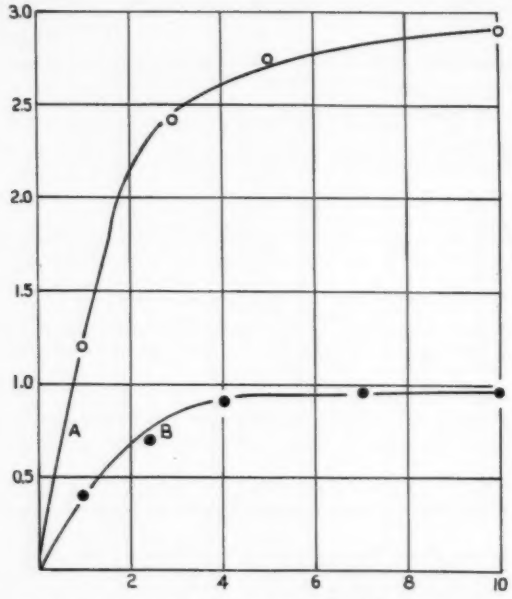

FIG. 4. Hydrolysis of tributerine by lipase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 NaOH; the abscissa is the time in hours.

DISCUSSION

The experiments show the presence of a proteinase, amylase, and lipase which partially complete the enzyme mechanism for handling proteins, carbohydrates, and fats available to *Daphnia* in its natural habitat and under natural feeding conditions. These experiments show that the activities of the proteinase and amylase are such that considerable digestion is possible in the 15–30 minutes that food is said to remain in the alimentary tract. From Fig. 1 it was computed that 88 per

cent of the total digestion of gelatin done by a 1 per cent extract of *Daphnia* was completed within 20 minutes. Fig. 3 shows that of the total amount of maltose formed by amylase in 75 minutes, 80 per cent was produced in the first 30 minutes.

Figure 1 illustrates a comparison of the proteolytic activity of a 1 per cent glycerol extract of defatted *D. pulex* and a 1 per cent glycerol extract of defatted hog pancreas. It is apparent that the hog pancreas extract contains more enzyme than the *Daphnia* extract, due primarily to the fact that the digestive enzymes of the hog are most concentrated in the pancreas. On the other hand, however, if the amount of proteinase present per body weight of *Daphnia* be compared with the amount per body weight of hog, the picture would look very different. Estimating a hog to weigh 200 lbs., the hog pancreas 0.5 lbs., and the average *Daphnia* 0.0043 oz., rough calculations approximate the amount of proteinase to be from 300 to 400 times greater per body weight of *Daphnia* than per body weight of hog.

The proteinase obtained by gross extraction of the entire organism is found to have an optimum digestion at pH 7.4. This extract may obviously contain proteases other than the digestive enzymes, and any interpretation of the results may be made with this in view. Isolation of a proteinase from the digestive tract of *Daphnia* was not attempted. On the other hand, the extracts of 50 isolated digestive tracts of *D. magna* showed definite proteolytic activity at pH 7, which corresponds with the pH observed in the digestive tract and with the optimum pH of the enzyme mixture obtained from the entire animal. It will be recalled that mammalian tissue proteases react best in slightly acid media-about pH 4 +, and not at all at 7. The author believes, therefore, that he is warranted in assuming that the proteinase obtained by extracting the entire *Daphnia,* acting best at pH 7.4, is the digestive enzyme of the alimentary tract.

The character of the proteinase simulates that of mammalian trypsin and the initial cleavages on gelatin were undoubtedly produced by this tryptic-like proteinase, since Waldschmidt-Leitz (1929) holds that erepsin does not hydrolyze gelatin. It is very probable, however, that erepsin was present and played some part in the splitting of the poly- and di-peptides after the initial cleavages by the proteinase had been made. In addition, the fact that the extract was practically inactive at pH 3.8 and completely inactive at 3.2 rules out the possibility of the presence of a peptic type of digestion.

Summary

1. A digestive enzyme system exists in *Daphnia* that enables it to digest considerable protein and carbohydrate within 30 minutes.

2. Microchemical analysis of glycerol extracts of *D. magna* intestines demonstrated the presence of a proteinase similar to trypsin. Quantitative estimations were made of proteinase activity of whole *D. pulex* extracts.

3. No pepsin was found in the extracts.

4. Amylase and lipase were chemically demonstrated in extracts of *Daphnia.*

5. The pH of the alimentary tract of *D. magna* was found to range from 6.8–7.2.

LITERATURE CITED

BAKER, J., AND H. F. E. HULTON, 1920. The Iodimetric Estimation of Sugars. *Biochem. Jour.,* **14**: 754.

BIRGE, E. A., AND C. JUDAY, 1922. The Inland Lakes of Wisconsin. The plankton. 1. Its quantity and chemical composition. Bull. No. 64. Wis. Geol. and Nat. Hist. Survey.

BIRGE, E. A., AND C. JUDAY, 1934. Particulate and Dissolved Organic Matter in Inland Lakes. *Ecol. Mon.,* **4**: 440.

GATES, F. L., 1927. A Method of Proteolytic Enzyme Titration. *Proc. Soc. Exper. Biol. and Med.,* **24**: 936.

KLUGH, A. B., 1927. The Ecology, Food-Relations and Culture of Fresh-Water Entomostraca. *Trans. Roy. Can. Inst.,* **16**: 15.

KROGH, A., 1930. Über die Bedeutung von gelösten organischen Substanzen bei der Ernährung von Wassertieren. *Zeitschr. vergl. Physiol.,* **12**: 668.

NAUMANN, E., 1918. Über die natürliche Nahrung des limnischen Zooplanktons. *Lunds Univ. Arsskrift* N.F. Avd. 2, **14** (No. 31) : 1.

NAUMANN, E., 1921. Spezielle Untersuchungen über die Ernährungsbiologie des tierischen Limnoplanktons. 1. Über die Technik des Nahrungserwerbs bei den Cladoceren und ihre Bedeutung für die Biologie der Gewässertypen. *Lunds Univ. Arsskrift.* N. F. Avd. 2, **17** (No. 4) : 1.

NORTHROP, J. H., AND R. G. HUSSEY, 1922. A Method for the Quantitative Determination of Trypsin and Pepsin. *Jour. Gen. Physiol.,* **5**: 353.

PÜTTER, A., 1909. Die Ernährung der Wassertiere und der Stoffhaushalt der Gewässer. Gustav Fischer. Jena.

RANKIN, G. P.,1929. The Nutritional Physiology of Cladocera. *Cont. Canad. Biol. and Fish.,* **4** (No. 8) : 109.

STUART, C. A., M. MCPHERSON, AND H. J. COOPER, 1931. Studies on Bacteriologically Sterile Moina macrocopa and their Food Requirements. *Physiol. Zool.,* **4**: 87.

WALDSCHMIDT-LEITZ, E., 1929. Enzyme Actions and Properties, p. 147. John Wiley and Sons.

THE EFFECT OF CUPRIC, MANGANOUS, AND FERRIC CHLORIDES UPON CARDIAC EXPLANTS IN TISSUE CULTURE

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Introduction

Considerable material has appeared in the literature concerning the effect of many metallic ions and salts upon various biological functions. It has appeared from the studies of Titus and Cave (1928), Titus, Cave and Hughes (1928) and Elvehjem (1932) that manganese and iron are particularly prominent in numerous rôles, especially in cellular respiration, hemoglobin formation, and nutrition. To introduce a third element, copper likewise is essential to hemoglobin formation regardless of the amount of iron administered to the organism (Titus and Cave, 1928). Further, it was shown by Titus and Hughes (1929) that copper and manganese may be stored in the animal body in such a way as to be effective in the utilization of iron in respiratory pigment formation. Later studies by Locke and Main (1931) have provided additional evidence that iron is essential for the regulation of cellular respiration and that it, together with copper, forms the oxygen-binding nucleus of the respiratory pigments. The absorption of oxygen by the cells is due to a reaction between molecular oxygen and a complex intracellular iron compound which is believed to be a hematin derivative and is termed "the respiratory enzyme" (Warburg, 1923*a*, 1923*b*, 1925, 1926, 1928; Warburg, Posener and Negelein, 1924).

The fact that these metals play a further part in the proper function of the blood is brought out by Meyer and Eggert (1932), who reported beneficial results following the administration of copper and iron combinations in the treatment of secondary anemia, while the liver and liver extracts used in primary anemia have appeared to be effective for reasons other than that they contain high percentages of these metals. On the other hand, the beneficial effects of hepatic therapy in secondary anemia may be due, at least in part, to the presence of these two metals.

The absence of iron and copper from synthetic media results in the slowing up of yeast cell proliferation and the production of atypical cells with low pigment content. This reduction of pigment has been held to be comparable, in certain respects, to the condition of anemia in animals (Elvehjem, 1931).

Leaving the question of iron and copper for the moment and turning to that of manganese, it has been shown that horses and goats, immunized against diphtheria, and exhibiting a constant fall of antitoxin titre, would increase the titre following injections of manganese chloride. Small injections of this salt increased the power of the organism to destroy the bacterial toxins to such a degree that animals so treated were not poisoned by an otherwise fatal dose of toxin. This was particularly true in cases of tuberculosis in mice and guinea pigs (Walbum, 1921, 1924). Although manganese will raise the titre of diphtheria antitoxin, it will not change that of tetanus, nor has it any effect upon agglutinins or hemolysins (Pico, 1924).

The experiments reported in the present article were devised to test the effect of different concentrations of the three metals mentioned upon the growth and longevity of cells in tissue cultures. The 3,000 cultures examined in this study have yielded some rather definite information on the questions of the toxicity of, and the tolerance to, these elements when used singly and in combinations. It became of interest to determine whether a tolerance could be built up for such solutions as proved toxic; and whether a combination of non-toxic, or even beneficial solutions was more beneficial than the constituents used separately.

Technique

All of the cultures used in these experiments were made by the cover-slip-hanging-drop method. The procedures were carried out with aseptic precautions in each instance. All water used was triply distilled in an all-Pyrex-glass apparatus. The plasma was obtained by centrifuging blood drawn from the wing veins of young hens. Embryo juice (33 per cent) was prepared by extracting seven-day, or eight-day chick embryos in Tyrode solution (pH 7.4 to 7.6) containing 0.25 per cent dextrose. In order to increase the potency of the extract, it was allowed to stand twenty-four hours at 40° F. before centrifuging (Carrel, 1913). In the experimental series the metallic salts (Merck's), in sufficient quantities for the proper final dilutions, were added to the embryo juice before mixing. Tissue from the hearts of seven-day, or eight-day chick embryos was planted in a mixture of equal quantities of plasma and embryo juice containing the metallic salt. An equal number of controls, using the same plasma, stock embryo juice, and embryo heart tissue, was run with each series of the metallic chloride cultures. These cultures were incubated at 39° C. and careful daily records were kept on each as long as it remained alive. An average percentage death curve was constructed from the data obtained in the control cultures of all experiments

in this paper and serves as the standard for comparison with the curves obtained from the metallic ion series.

Experimental

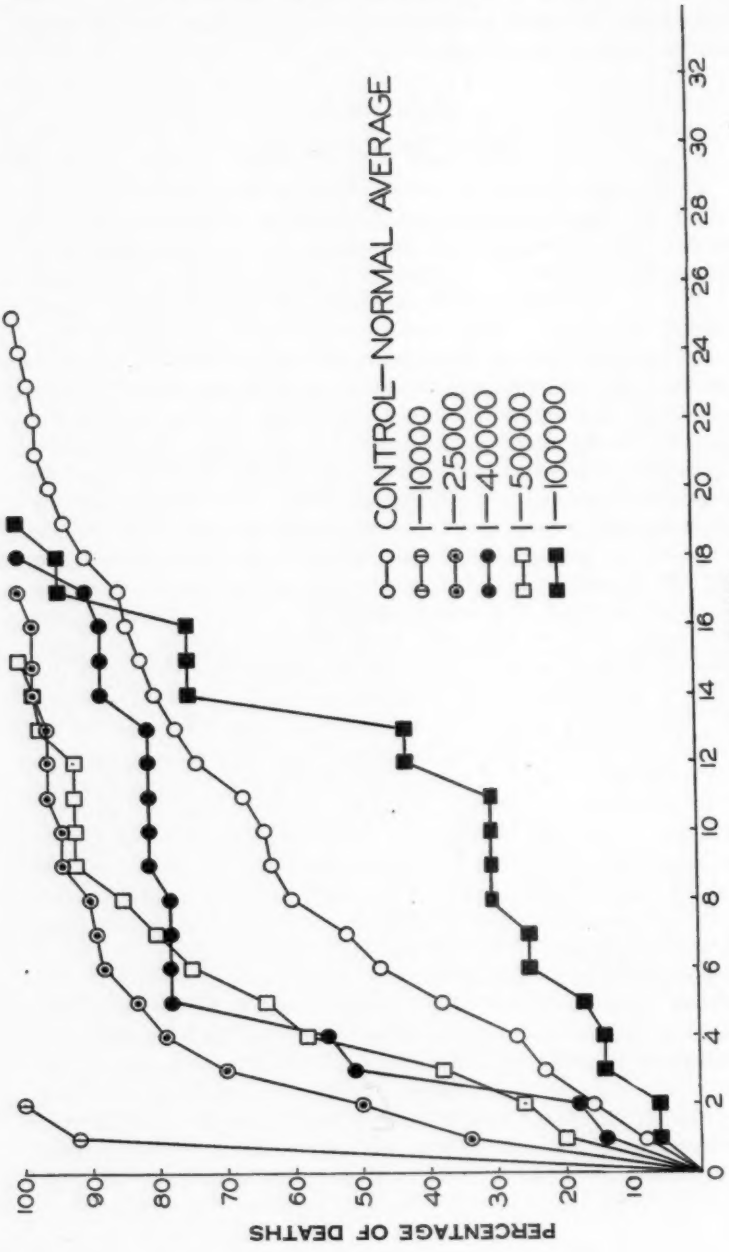
Cupric Chloride Experiments

In these experiments to test the effect of the copper salt, $CuCl_2 .2H_2O$ was added to embryo juice samples in sufficient quantities to make a range of dilutions and normalities, after an equal quantity of plasma had been added, as follows: 1–10,000 (.00117 N), 1–25,000 (.00056 N), 1–40,000 (.00029 N), 1–50,000 (.00023 N), 1–100,000 (.000117 N).

The average death curve of the control cultures climbed rapidly at first and then more gradually until the twenty-fifth day when all cultures were dead. Cupric chloride—1–10,000 (Graph I)—was found to be very toxic to the explants, as evidenced by an almost straight curve; 92 per cent of all the cultures died within twenty-four hours and the remaining 8 per cent within forty-eight hours. In cultures with this concentration of copper, growth was practically inhibited; only occasionally was there any evidence of the migration of fibroblasts. In the 1–25,000 dilution series a fair radial outgrowth of mesenchymal cells was present twenty-four hours after planting. However, the curve rises very rapidly until the sixth day, after which it resembles the control curve. This may be due to either of two factors or a combination of them. It is possible that the tissues became accustomed to the copper salt or, after active proliferation of fibroblasts began, the toxicity of the chloride may have become proportionately less as the cell mass increased.

The curves of cultures planted in 1–40,000 and 1–50,000 dilutions of the copper salt rise rapidly and more or less in company until the fifth day. Then they diverge and the lesser dilution curve flattens out, rising slowly until the seventeenth day. On the other hand, the 1–50,000 dilution curve continues to rise until the ninth day, after which the rise is less acute until the fifteenth day when all cultures were dead. The radial outgrowth of cells in both these dilutions was good, though markedly better in the greater dilution. This fact may account for the different nature of the curve. The possibility, which suggests itself here, is that the life span was shorter because of lack of nutriment.

In the 1–100,000 cupric chloride series, the radial outgrowth of the cells was very marked and exceeded the growth of the controls. This is evidenced by the curve which shows that on the eleventh day only 30.5 per cent of the cultures were dead as compared with 67 per cent of the controls. However, from this point on, the curve rises very



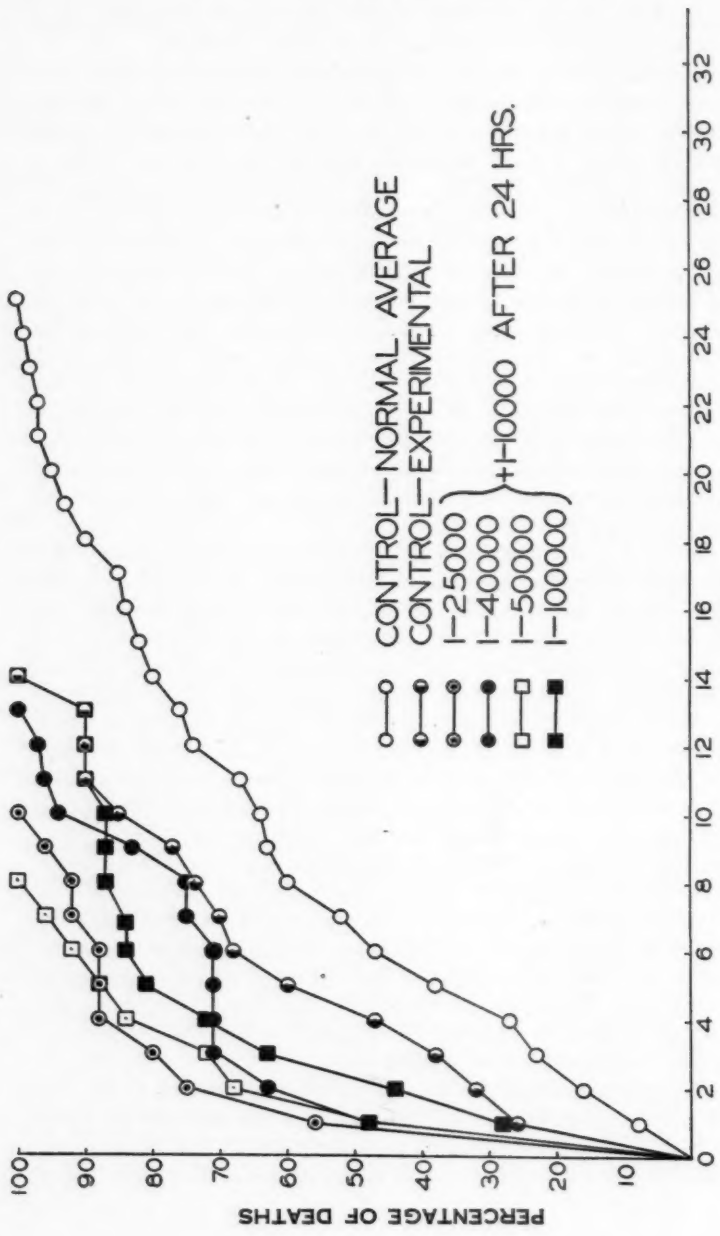
Graph I. Cupric chloride dilutions.

rapidly, possibly because of the toxic effect of the salt together with the lack of nutriment and accumulation of waste products.

A second series of experiments to test a possible tolerance of the cells to cupric chloride was carried out by the administration of a toxic concentration of the metallic salt after a period of adjustment in greater dilutions. Cultures were planted in each of the four salt dilutions: 1–25,000, 1–40,000, 1–50,000 and 1–100,000 and allowed to incubate for twenty-four hours. At the end of this period of preliminary adjustment and growth, each living culture was unsealed, the fluid drawn off and replaced by one drop of 1–10,000 copper chloride embryo juice. This concentration was selected because it had been found to be the most toxic of the solutions employed. Following this treatment the cultures were sealed, incubated as usual, and observed at twenty-four hour intervals until all were dead. The experimental controls for this series consisted of cultures planted in the regular manner without any copper salt added until after the first twenty-four hours of incubation when one drop of 1–10,000 copper chloride embryo juice was added. Thereafter the results were recorded as for the other series and all appear in Graph II.

For the first three days the percentage death curves have much the same shape, rising rapidly. During this interval the toxicity was most marked in the 1–25,000 dilution (80 per cent), about the same for 1–40,000 and 1–50,000 (71 and 72 per cent), less in the 1–100,000 (63 per cent) and least in the experimental control (38 per cent). If one compares the curve of cultures planted in 1–10,000 $CuCl_2$ (Graph I) with the curves of the culture series in the varying dilutions to which 1–10,000 $CuCl_2$ was added after twenty-four hours (Graph II), one notes that the additional copper is tolerated rather well though the life span is much less (a difference of eleven days for 1–100,000 $CuCl_2$ series) for all series compared with that of the normal controls. The experimental controls, which were normal cultures allowed twenty-four hours undisturbed growth and then subjected to copper chloride 1–10,000, died off less rapidly than the cultures planted in the various dilutions and then treated with the toxic dose. The total life span, however, was the same as that of the 1–100,000 dilution cultures in this particular experiment. It seems from this that a very critical period in the life of the explanted tissues occurs within the first twenty-four hours of adjustment and growth.

Another experiment was devised to test whether the age of the culture, before adding the toxic 1–10,000 $CuCl_2$, would influence the tolerance as evidenced by the longevity of the tissue. A large series of cultures was planted in the various dilutions of copper chloride as before



GRAPH II. Cupric chloride tolerance.

and divided into groups. After twenty-four hours incubation a set of vigorous cultures from each dilution series was unsealed and each preparation received one drop of 1–10,000 $CuCl_2$ embryo juice. They were resealed and returned to the incubator. On the second, third, fourth and fifth days a fresh group from each series was similarly treated. By this means the cultures were allowed to establish growths in their respective copper dilutions from one to five days before their susceptibility to the toxic concentration of copper was tested.

The results of this experiment were rather unsatisfactory. However, the series in which the procedure duplicated that of the tolerance experiment just described, i.e., addition of the toxic solution on the day following planting, reproduced the curves of Graph II almost exactly. Two days difference in the life span was the greatest variation and that occurred in the 1–50,000 dilution.

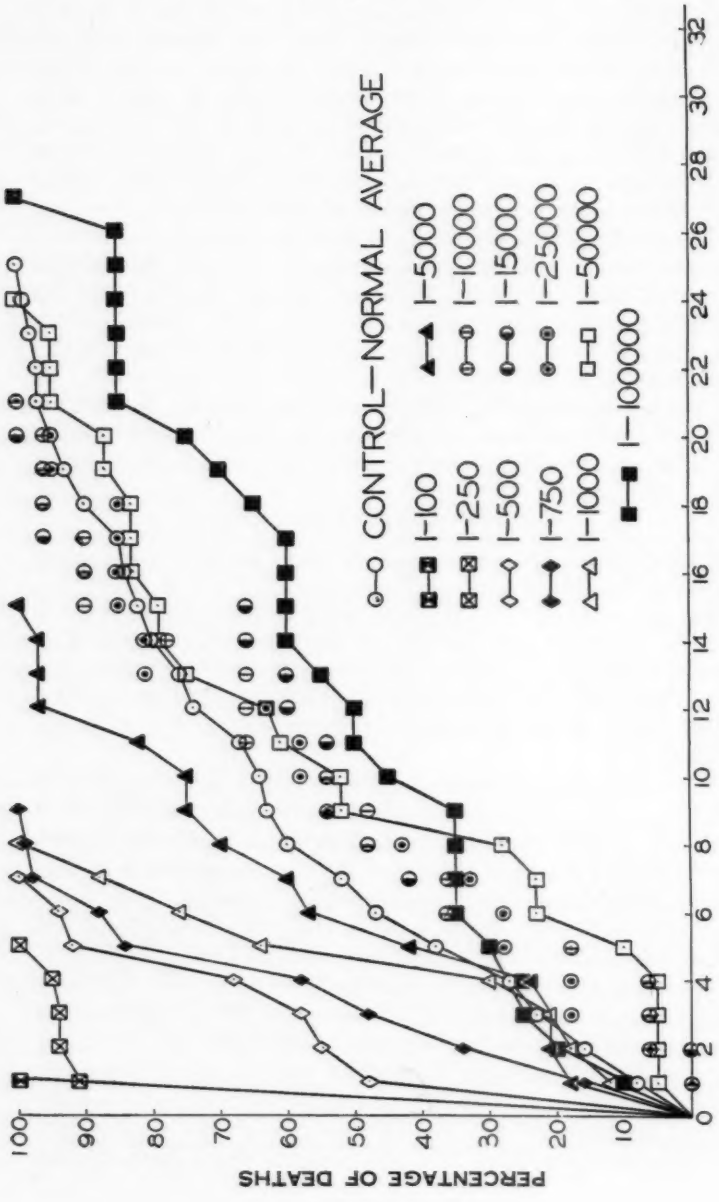
With the exception of the cultures in 1–25,000 dilution, the optimum resistance to an added toxic dose of copper salt, as evidenced by plotting the results, was acquired between the third and fourth days of growth.

Manganous Chloride Experiments

Experiments, conducted in a manner similar to those of the copper series, were carried out with manganous chloride, $MnCl_2.4\ H_2O$, added to the embryo juice. This salt was added in quantities sufficient to form the following final dilutions in which series of cultures were planted: 1–100 (.10 N), 1–250 (.04 N), 1–500 (.02 N), 1–750 (.013 N), 1–1,000 (.010 N), 1–5,000 (.002 N); 1–10,000 (.0001 N), 1–15,000 (.0006 N), 1–25,000 (.0004 N), 1–50,000 (.0002 N), 1–100,000 (.0001 N).

When the percentage death curves were plotted and compared with the average curve of all normal controls, it was found (Graph III) that they fell roughly into three major groupings. The series 1–100 through 1–1,000 showed a marked toxicity of the salt; the 1–5,000 sets were isolated midway between these and the remaining dilutions. The curves of all dilutions from 1–15,000 through 1–50,000 remained below the control curve until the twelfth day after which they fell to one side or the other of this curve. The 1–100,000 dilution curve, from the fourth day onward, was always below that of the control and terminated on the twenty-seventh day, exceeding the control in length by two days.

From these curves it will be seen that the presence of manganous chloride in dilutions of 1–15,000 through 1–50,000 did not markedly influence the behavior of cells in cultures although the life span was decreased one to five days. The cells grew and radial outgrowth was



GRAPH III. Manganous chloride dilutions.

almost as prolific as in the controls The aging process,[1] slower in these cultures than in the controls, was hastened a little after the twelfth day.

Manganous chloride 1–100,000 prolonged the life of cultures for two days as compared with the controls. This in itself was not a significant increase, but in view of the fact that the percentage deaths throughout the series on any one day after the sixth were from 10 to 20 per cent less than for the normal controls, it seemed that manganese in this concentration may have exerted a slight influence in the direction of longevity.

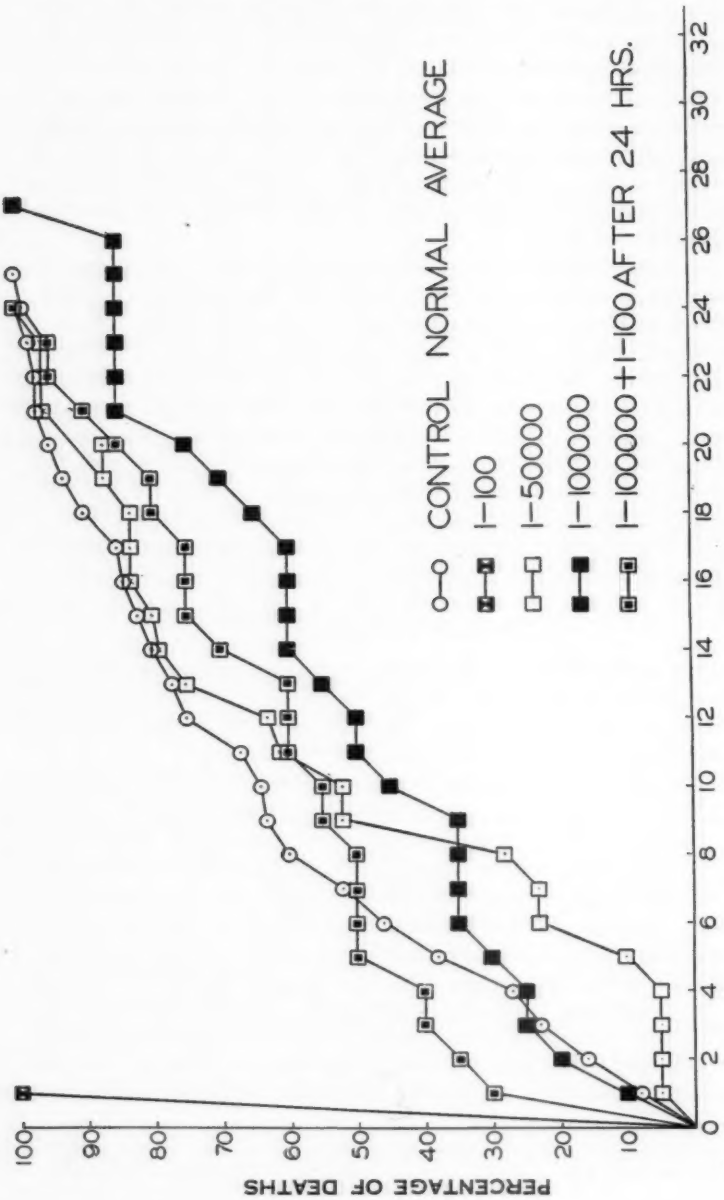
To ascertain whether the cultures would develop a tolerance for manganese, a series of plantings in 1–100,000 dilution were allowed to adjust themselves for twenty-four hours. At the end of that time they were unsealed and to each culture was added one drop of 1–100 $MnCl_2$ embryo juice. Then they were resealed and returned to the incubator. The results of these series showed that the addition of the very toxic solution (see Graph IV) did not cause the death curve to rise precipitously as in the 1–100 dilution series, but rather to rise rapidly until the tenth day and thereafter to follow very closely the curve of the 1–50,000 dilution. It would seem, therefore, that preliminary treatment with a non-toxic manganese concentration protected, to some extent, cultures subjected later to a fatal concentration.

Ferric Chloride Experiments

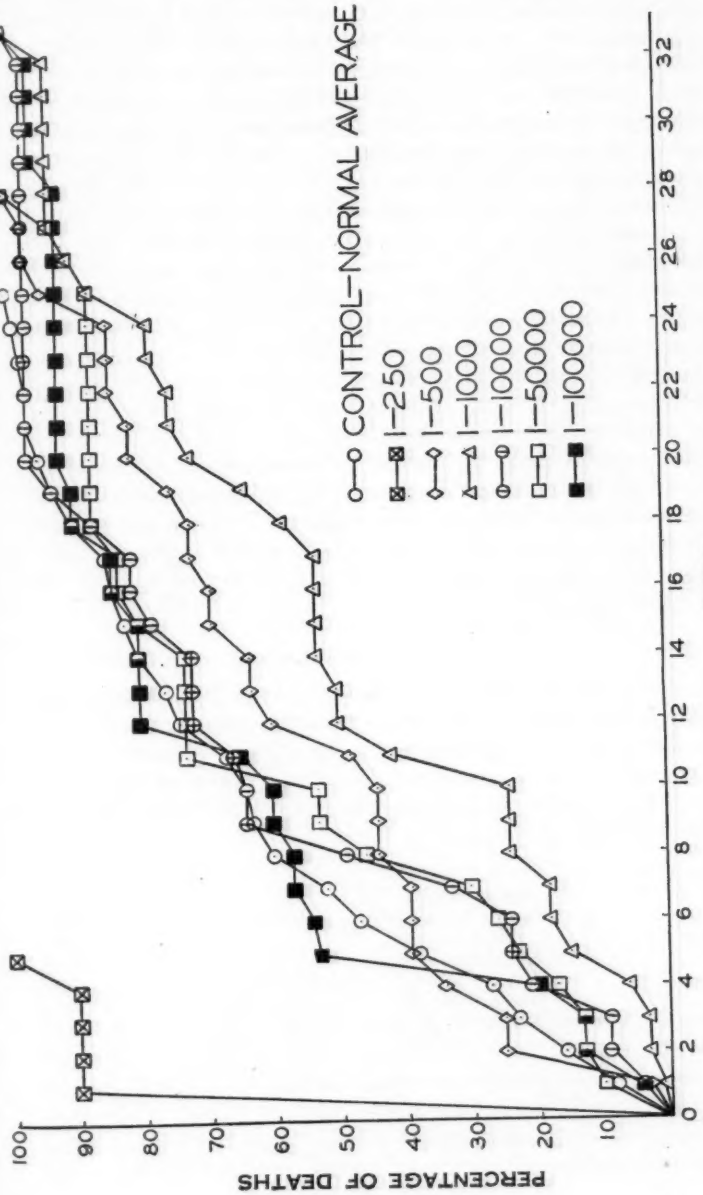
In a manner similar to that used in previous experiments plantings were made in the following $FeCl_3.6\ H_2O$ solutions: 1–250 (.044 N), 1–500 (.022 N), 1–1,000 (.011 N), 1–10,000 (.0011 N), 1–50,000 (.00022 N), 1–100,000 (.00011 N).

The results of these series are shown in Graph V. The dilution 1–250 proved to be highly toxic, 90 per cent of the cultures died in the first twenty-four hours and all of them were dead in five days. The curves of the other dilutions except 1–500 and 1–1,000 followed quite closely that of the average normal control, falling but a short distance above or below it until the eighteenth day when they diverged. With the exception of the 1–250 dilution, all other curves exceeded the normal in duration from three to eight days. Contrary to the results obtained for the copper and manganese chlorides, the 1–1,000 dilution of the $FeCl_3$ proved to be the least toxic of all and the life span of this series was prolonged eight days over that of the controls. This is a significant increase and might suggest that this dilution of ferric chloride was

[1] The addition of potassium permanganate to mesenchymal cells in cultures was found by Lewis (1921) to reproduce, within a few minutes, the degenerative changes that took place gradually in the normal aging and resultant death of cells.



GRAPH IV. Manganous chloride tolerance.



GRAPH V. Ferric chloride dilutions.

beneficial to cultures. However, one observation must be kept in mind in this connection. After tissues were planted in the $FeCl_3$ solutions, a brown precipitate was produced in the medium and in all the iron salt cultures, histiocytes were found in noticeably greater numbers than in either copper or manganese cultures. These phagocytic cells contained quantities of engulfed iron precipitates. Whether the formation of precipitates rendered the iron less toxic or whether the abundance of histiocytes exerted a beneficial influence upon the cultures is not known. As suggestive evidence, only, in support of the latter idea, the senior author noted, in some other experiments using cardiac tissue planted in a medium containing trypan blue, that histiocytic outgrowth was stimulated and that cultures so populated remained in better condition longer than those without an abundance of macrophages.

In order to test the development of tolerance to iron, a series of cultures was planted in 1–1,000 $FeCl_3$ and incubated for twenty-four hours before each culture received one drop of 1–250 iron salt. As in previous experiments of this nature, the preliminary treatment of the cultures with the least toxic dilution of the salt lessened (Graph VI) to a marked degree the toxicity of a fatal dose; although the life span was reduced from thirty-three days (1–1,000 dilution curve) to twenty-six days (tolerance curve) as compared with the five-day span for cultures planted only in 1–250 dilution.

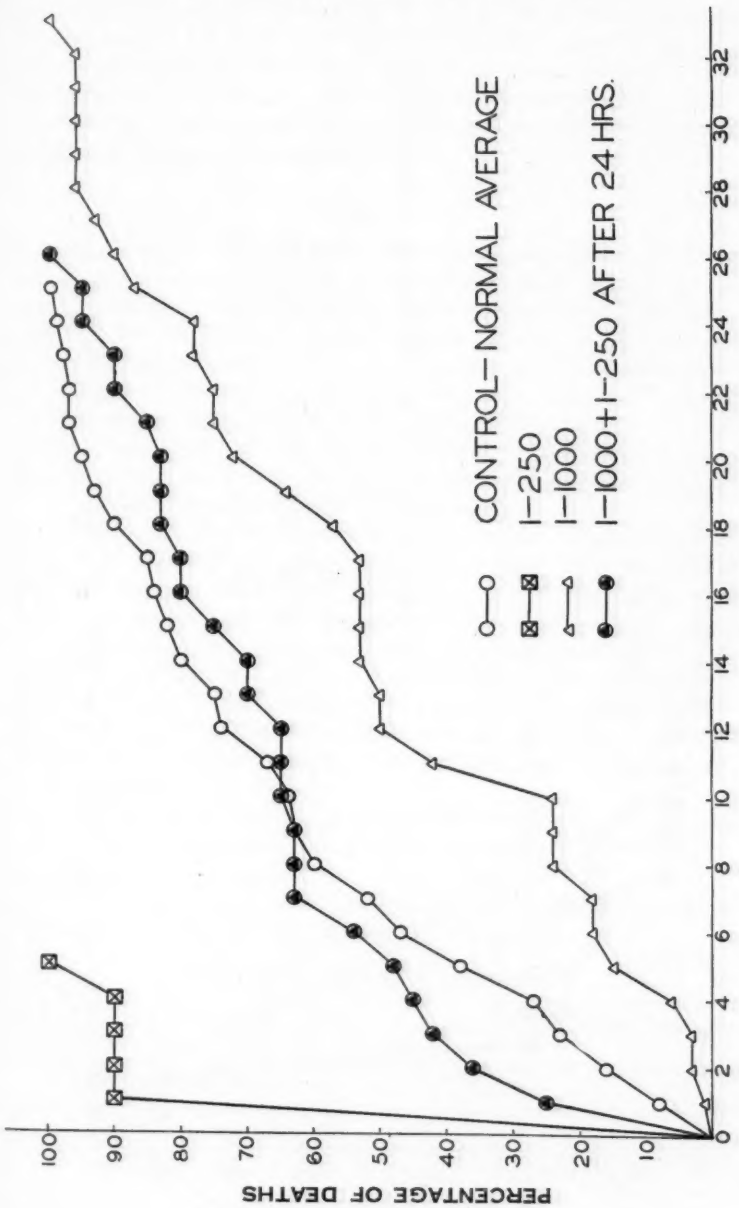
Combination Experiments

In view of the fact that certain dilutions of each of the three metallic chlorides utilized in the preceding experiments were found to be relatively non-toxic, four combinations of the optimum dilutions of the different chlorides were made and cultures were planted in them. The same technique used in the previous experiments was employed here.

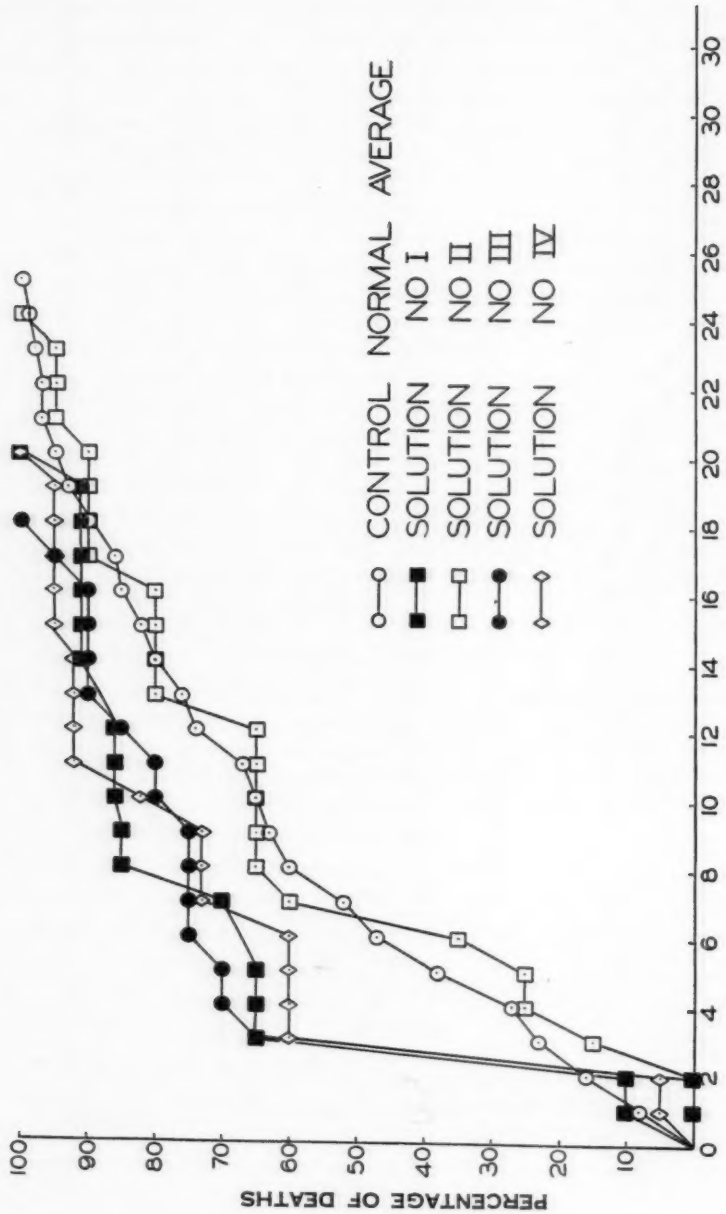
The salt mixtures used were as follows:

Solution 1.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
	(*c*) Manganous Chloride	1–100,000
Solution 2.	(*a*) Ferric Chloride	1–1,000
	(*b*) Manganous Chloride	1–100,000
Solution 3.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
Solution 4.	(*a*) Manganous Chloride	1–100,000
	(*b*) Cupric Chloride	1–100,000

Solutions 1, 3, and 4 were the most toxic and caused sudden death of 60 to 65 per cent of the cultures by the third day (Graph VII); those remaining died by the eighteenth to twentieth days as compared with a life span of twenty-five days for the normal controls. Solution 2 was



GRAPH VI. Ferric chloride tolerance.



GRAPH VII. Combination solutions.

the least deleterious; its curve followed closely that of the control and the life span of the series was only one day less. None of the salt mixtures had any advantage over the single optimum dilutions of the respective salts composing it. It was noted again, however, that wherever iron was present in the solutions, the cultures contained a greater number of histiocytes than otherwise.

Summary

Throughout these experiments there were certain characteristic reactions in the mesenchymal cells growing in the various dilutions of the metallic chlorides used. The nuclear membrane and nucleoli were generally a little more sharply defined than in the cells of the controls. Accumulation of vacuoles, first about the nucleus and finally throughout the cytoplasm, took place rapidly in the toxic salt solutions. At times the cells were so thoroughly occupied by fatty looking vacuoles that they bulged, rounded up and floated away. Similar accumulations of vacuoles occurred in the controls but their appearance was gradual and they only assumed great numbers as the cultures aged.

Optimum dilutions of the metallic salts, in which a depression of the daily death rate and an increase in the life span of the cultures were noted, delayed the degenerative changes in the cells beyond the time when they usually made their appearance in the controls.

Of these solutions, cellular outgrowth was actually stimulated in that of copper chloride, the death rate was depressed in that of manganese chloride, as also in that of iron chloride. Furthermore, the life span was increased in the latter a significant number of days. This effect possibly may have been brought about indirectly as a result of histiocytic stimulation.

Tissues could be protected to some extent from the action of a fatal dose of these three metallic salts by first growing the cells in the respective optimum dilutions.

No beneficial effects were obtained by growing tissues in various combinations of the optimum dilutions of these salts.

Literature Cited

Carrel, A., 1913. Artificial Activation of the Growth in Vitro of Connective Tissue. *Jour. Exper. Med.*, **17**: 14.

Elvehjem, C. A., 1931. The Rôle of Iron and Copper in the Growth and Metabolism of Yeast. *Jour. Biol. Chem.*, **90**: 111.

Elvehjem, C. A., 1932. The Relative Value of Inorganic and Organic Iron in Hemoglobin Formation. *Jour. Am. Med. Ass.*, **98**: 1047.

Lewis, Warren H., 1921. The Effect of Potassium Permanganate on the Mesenchyme Cells of Tissue Cultures. *Am. Jour. Anat.*, **28**: 431.

LOCKE, A., AND E. R. MAIN, 1931. The Relation of Copper and Iron to Production of Toxin and Enzyme Action. *Jour. Infect. Dis.*, **48**: 419.

MEYER, A. E., AND C. EGGERT, 1932. Iron and Copper in Liver and Liver Extracts. *Jour. Biol. Chem.*, **99**: 265.

PICO, C. E., 1924. Influence du manganèse sur les phénomènes de l'immunité. *Compt. rend. Soc. Biol.*, **91**: 1049.

TITUS, R. W., AND H. W. CAVE, 1928. Manganese as a Factor in Hemoglobin Building. *Science*, **68**: 410.

TITUS, R. W., H. W. CAVE, AND J. S. HUGHES, 1928. The Manganese-copper-iron Complex as a Factor in Hemoglobin Building. *Jour. Biol. Chem.*, **80**: 565.

TITUS, R. W., AND J. S. HUGHES, 1929. The Storage of Manganese and Copper in the Animal Body and Its Influence on Hemoglobin Building. *Jour. Biol. Chem.*, **83**: 463.

WALBUM, L. E., 1921. Action exercée par le chlorure de manganèse et d'autres sels métalliques sur la formation de l'antitoxine diphtérique et l'agglutinine du B. coli. *Compt. rend. Soc. Biol.*, **85**: 761.

WALBUM, L. E., 1924. Therapeutic Experiments with Metal Salts. *Acta pat. et microbiol. Scandinav.*, **1**: 378.

WARBURG, O., 1923*a*. Versuche an überlebendem Carcinomgewebe. *Biochem. Zeitschr.*, **142**: 317.

WARBURG, O., 1923*b*. Über die Grundlagen der Wielandschen Atmungstheorie. *Biochem. Zeitschr.*, **142**: 518.

WARBURG, O., 1925. Bemerkung zu einer Arbeit von M. Dixon und S. Thurlow sowie zu einer Arbeit von G. Ahlgren. *Biochem. Zeitschr.*, **163**: 252.

WARBURG, O., 1926. Über die Wirkung von Blausäureäthylester (Äthylcarbylamin) auf die Pasteursche Reaktion. *Biochem. Zeitschr.*, **172**: 432.

WARBURG, O., 1928. Wie viele Atmungsfermente gibt es? *Biochem. Zeitschr.*, **201**: 486.

WARBURG, O., K. POSENER, AND E. NEGELEIN, 1924. Über den Stoffwechsel der Carcinomzelle. *Biochem. Zeitschr.*, **152**: 309.

THE NUTRITION OF COPEPODS IN RELATION TO THE FOOD-CYCLE OF THE SEA

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The importance of copepods as the chief source of food for several types of commercially valuable fish and whales is well known (Hardy, 1924; Wimpenny, 1929; Hjort and Ruud, 1929; Savage, 1931; and Campbell, 1934). Many other fish and various invertebrates depend upon copepods for food directly or indirectly. Since copepods derive their nourishment from more primitive organisms, their rôle may be regarded as that of "middle-man" in the main food-chain of the sea (Clarke, 1934*a*). The nutrition of copepods is, however, a matter about which very little is definitely known. The experiments described in this paper were therefore undertaken in an attempt to discover the precise organisms upon which copepods feed. This information is desired not only in relation to their growth but also in relation to their distribution (Clarke, 1934*b*). Furthermore, the development of a suitable method of culturing copepods in the laboratory has long been desired in order that the effects of temperature, light, and other important factors upon the growth and behavior of the animals could be investigated under carefully controlled conditions. To accomplish this a knowledge of the food of copepods is obviously a primary prerequisite.

Previous Investigations

Previous investigations of the food of copepods have been largely inconclusive, as already pointed out (Clarke, 1934*a*; cf. also Yonge, 1931). In general the observations may be divided into those which are claimed to indicate that copepods live on diatoms and those which appear to indicate that other organisms are the chief source of food. Examinations of the alimentary canals of copepods carried out by Dakin (1908), Esterley (1916), Marshall (1924), and Lebour (1922) revealed that a major part of the recognizable material was composed of diatom fragments. Crawshay (1915) kept *Calanus finmarchicus* alive in a pure culture of the diatom, *Nitzschia closterium*, for several weeks. Campbell (1934) believes that diatoms are the chief source of

[1] Contribution No. 66.

food for *Calanus tonsus* in the Strait of Georgia. She states, however, "It does not seem so essential that a large supply of food be available while the copepod is in the adult stage. During the fifth copepodid stage there is probably sufficient food stored as oil to tide the organism over the egg-producing period. . . . The very young stages of the copepod probably do not depend as much as the older stages upon the diatoms for food. Minute Protozoa and bacteria doubtless form the chief constituents of their diet."

Marshall, Nicholls, and Orr (1934) report that in Loch Striven during 1933 the periods of diatom increases coincided with the three main spawning periods of *Calanus finmarchicus*. They assert: "It is, of course, impossible to state what are the actual requirements of a *Calanus*, but the younger stages being less mobile, will require a richer supply than the later stages and adults. The critical time is therefore the period from egg to early copepodite, and the presence or absence of diatoms in the water then, means the success or failure of a brood." In addition they found that "The remaining constituents of the microplankton (chiefly minute flagellates) although at times numerous show no relation to breeding periods or survival." Thus Marshall, Nicholls, and Orr feel that diatoms are essential to the nauplii whereas Campbell believes that diatoms are an important source of food only during the late copepodid stages.

In contrast to these observations are those of Bigelow (1926), Johnstone (1911), and Fish (1925), in which an unmistakable decrease in the amount of zoöplankton was found to take place whenever diatoms were abundant. Fish's explanation is that "the common species [of diatoms] having these swarming periods do not form the food of the zoöplankton so far as I have been able to determine," and that "During the maxima of the large diatoms the smaller members of this group which are eaten by pelagic animals disappear, causing a scarcity in the food supply."

In at least two previous cases copepods have been kept in the laboratory on food other than diatoms. Murphy (1923) reared *Oithona nana* in small Stender dishes in each of which a small piece of fresh kelp was placed. Although a fair growth of *Navicula* and small Protozoa occurred, the copepods were observed to eat the kelp in large amounts. Bond (1933), using a culture of the green flagellate, *Platymonas*, as a source of food, reported that *Calanus finmarchicus* lived for over two months, *Euchaeta* sp. for over five weeks, and *Tigriopus fulvus* for several months including many generations. Bacteria were not excluded from the culture media in either of these cases nor in the experiments of Crawshay cited above. Since copepods appear to be well

equipped with enzymes (Bond, 1934), their diet should not be limited from lack of powers of digestion.

Material and Methods

The following copepods obtainable within a three hours run of Woods Hole were used in the present investigation: *Centropages typicus* and *hamatus, Labidocera aestiva, Acartia tonsa,* and *Calanus finmarchicus.* Only about 100 specimens in several liters of water were brought in at one time and the catch was kept cold in the boat's ice box until the laboratory was reached. Immediately upon arrival the copepods were transferred by means of a large-mouthed pipette to the containers to be used for culturing. The containers were placed in one of two constant temperature tanks or in a large refrigerator. The tanks were maintained at different temperatures (kept constant to 0.1° C.) by means of two Kelvinator cooling units operated by Hiergesell thermoregulators and relays.

The sea water used in the containers was taken from the laboratory tap except where otherwise specified. In certain experiments the water was changed by pouring away all but a little of the original culture medium, leaving the copepods in the bottom corner of the dish and then replenishing with fresh sea water. The effect of stirring and aëration was investigated by using beakers equipped with plungers activated by the Plymouth siphon device (Harvey, 1928) or by employing Erlenmeyer flasks through which compressed air from a tap filter pump was slowly bubbled.

The materials which were tested as possible sources of food for the copepods included, first, the organisms already present in the sea water. In a few experiments the water was centrifuged and the material thrown down added to the culture dishes. Material taken in the harbor with a diatom net was introduced into the containers in other cases. In a large number of the experiments "persistent"[2] cultures of diatoms and green flagellates grown in the laboratory were used. With the kind assistance of Professor H. H. Gran a culture of *Nitzschia closterium* was started from specimens obtained in the vicinity of Woods Hole. The cells grew chiefly on the bottom and sides of the vessel and the copepods provided with this culture did not survive. Since one supposes that filter-feeders such as copepods (Cannon, 1928) can consume material in suspension only, this culture and others of the encrusting type were abandoned as a food source. Entirely different was the culture of *Nitzschia closterium* kindly sent me by Dr. E. J. Allen from the Laboratory of the Marine Biological Association at Plymouth. In

[2] By a "persistent" culture is meant one which contains only one species of alga but is not free from bacteria and Protozoa (cf. Allen and Nelson, 1910).

this case the diatoms grew almost entirely in suspension and the cells themselves were quite dissimilar from the Woods Hole form, being smaller and almost straight. The following green flagellates were also found to remain in suspension in pure culture and were tried as sources of food: *Carteria mediterranea,* procured from the Pflanzenphysiologisches Institute der Deutschen Universität at Prague; *Chlamydomonas* sp., obtained from tide pools on Black Rock at the entrance of New Bedford Harbor;[3] and a mixed culture of *Dunaliella marina* and *D. salina,* kindly sent me by Dr. R. M. Bond.

TABLE I

The survival of *Calanus* in relation to the presence of diatoms and green flagellates. At 15° C., small crystallizing dishes were used each containing 2 copepods in 35 cc. of filtered harbor water and 5 cc. of the food culture. At 12° C., Erlenmeyer flasks were used each containing 5 copepods in 275 cc. of filtered harbor water and 25 cc. of the food culture.

Food material added	Copepods alive after the following no. of days:					Total number of molts
	0	5	11	19	25	
	Temperature: 15° C.					
Nitzschia	10	9	4	2	2	3
Dunaliella	10	9	7	4	3	3
Carteria	10	10	6	1	0	1
Chlamydomonas	10	10	5	1	1	3
No food added	10	9	3	0	0	0
	Temperature: 12° C.					
Nitzschia	10	10	8	8	7	10
Dunaliella	10	10	5	3	1	1
Carteria	10	8	7	7	6	4
No food added	9	8	3	2	2	2

The diatoms and flagellates were all cultured by the following method recommended to me by Professor H. H. Gran. Sea water from the laboratory tap was filtered, heated to 70° C., and allowed to cool. To each liter was added 10 cc. of the following nutrient solution: 0.1 per cent KNO_3 and 0.01 per cent Na_2HPO_4 in distilled water. Growth was improved by adding also to each liter of sea water 5–10 cc. of a soil extract made as follows: To 1 kg. of rich, dark garden soil add 1 liter of distilled water. Autoclave at a pressure of 15 lbs. for

[3] I am indebted to Professor W. R. Taylor for assistance in locating and identifying this organism.

30 minutes. Decant and filter. (Sterilize, if extract is not used immediately.) The diatom cultures were placed in a north window, the flagellate cultures in a south window but shielded from direct sunlight. When heavily inoculated, a good growth was obtained in large Pyrex Erlenmeyer flasks after about 10 days.

Experiments Using Diatoms and Green Flagellates

Preliminary experiments revealed that the copepods would die off rapidly if the temperature was allowed to rise above 20° C. or if the animals were overcrowded. When the culture water was not changed, an allowance of 20 cc. of water per copepod appeared to be adequate provided that the animals did not tend to cluster in one part of the culture dish. When provided with various of the food sources already mentioned (*Dunaliella, Chlamydomonas,* and *Carteria* were found to be equally efficacious), survival in the cases of *Centropages, Acartia,* and *Labidocera* was improved, but the majority of the animals did not live for more than about two weeks. In most of the cases in which the flagellates were added to the water, green material could be seen in the intestines of the copepods and many excretory casts were found on the bottom of the containers. Molted shells were observed only rarely. Stirring, aëration, and changing the culture water—thus avoiding the accumulation of metabolites—seemed to have no ameliorating effect. However, since we have not yet developed a completely successful method of keeping copepods alive in the laboratory, it is impossible to decide in many cases what the precise cause of the death of the animals was.

In the case of *Calanus finmarchicus* only a slight improvement in survival was found to result from the addition of green flagellates and diatoms to the culture water (Table I). The copepods, the majority of which were in copepodid Stages IV or V when taken, were examined every few days and any dead animals or molted shells were removed from the containers. Two specimens lived for 25 days in water to which nothing had been added. There appears to be no consistent difference in the effects of the several organisms tried as sources of food, but it is clear that more animals survived and more molted at 12° C. than at 15° C.

In a more elaborate experiment with *Calanus* (Table II) it was similarly found that the addition of *Dunaliella* did not enable a significantly larger proportion of the copepods to survive. However, this treatment resulted in a definitely increased number of molted shells. No significant difference appears to exist between the survival in sea water taken from the laboratory tap and in that taken directly from the

harbor. Of the two temperatures, the lower permitted a larger proportion of the copepods to survive but about the same total number of molted shells was observed. At the higher temperature growth would presumably be more rapid and hence a larger number of molted shells would be expected. However, since the period of ecdysis is known to be a critical one (cf. Hagmeier, 1930), it is possible that an increased rate of molting resulted in more deaths. Fewer animals would there-

TABLE II

The survival of *Calanus* in relation to the type of sea water with and without the addition of food material. Erlenmeyer flasks were used each containing 5 animals in 300 cc. water including, in the cases indicated, 25 cc. of the *Dunaliella* culture.

Source and treatment of water	Copepods alive after the following no. of days					Total no. of molts
	0	5	11	17	25	
	Temperature 15° C.					
Lab. supply, untreated, plus *Dunaliella*...	20	18	13	12	11	8
Lab. supply, untreated, no food added.....	5	5	3	3	3	5
Lab. supply, autoclaved, plus *Dunaliella*...	20	19	13	10	9	11
Harbor water, untreated, plus *Dunaliella*...	20	18	15	15	15	13
Harbor water, untreated, no food added...	5	5	3	3	3	2
Harbor water autoclaved, plus *Dunaliella*..	15	12	11	9	5	9
Harbor water autoclaved, no food added...	5	5	4	1	0	2
	Temperature 5–6° C.					
Lab. supply, untreated, plus *Dunaliella*....	20	19	19	18	16	15
Lab. supply, untreated, no food added.....	5	5	5	5	5	0
Lab. supply, autoclaved, plus *Dunaliella*...	15	13	11	10	10	7
Lab. supply, autoclaved, no food added....	5	5	5	2	2	1
Harbor water, untreated, plus *Dunaliella*...	20	19	19	18	18	19
Harbor water, untreated, no food added...	5	5	5	5	4	1
Harbor water, autoclaved, plus *Dunaliella*..	15	13	12	10	10	6
Harbor water, autoclaved, no food added..	5	5	4	4	4	1

fore be left to produce shells. A high mortality in connection with ecdysis might account in part for the better survival of the copepods at the lower temperature.

In this experiment an even larger proportion of the *Calanus* survived when no food material was added. This aroused the suspicion that the copepods were feeding on microörganisms or other material already present in the culture water. In every case, survival was better

in untreated water than in autoclaved water, and for the most part more shells were molted in the former medium. Although the sterilized culture water became contaminated immediately by bacteria introduced with the living copepods, the untreated water would presumably contain a richer supply of microörganisms. If the copepods could be shown to be able to derive nourishment from these organisms, the better survival in the untreated water would be explained. The possibility existed that in all of the experiments the green flagellates and diatoms were not being assimilated at all, or only to a slight extent, and that the ameliorating effect of adding these materials was due to

TABLE III

The survival of *Calanus* in relation to the presence of microörganisms.

Crystallizing dishes with 2 animals in each were used for the first and third sets of tests, Erlenmeyer flasks with 5 animals in each were used for the second set. Temperature 5–6° C.

Treatment of water (Harbor water)	Original no. of copepods	Copepods alive after 1 month
Berkefeld filtered	50	22
" " plus *Nitzschia*	47	8
Untreated	50	39
Untreated	15	9
Filtered through paper	15	9
Berkefeld filtered	15	0
Berkefeld filtered plus *Nitzschia*	15	8
Berkefeld filtered	40	0†
" " plus bacteria*	36	9†

* Culture of common forms taken from Woods Hole harbor and grown on agar slants kindly supplied by Dr. C. L. Carey.

† After three weeks.

the bacteria or other organisms which are present in "persistent" cultures (Waksman et al., 1933) and which might be used by the copepods as a source of food.

EXPERIMENTS USING MICROÖRGANISMS

To investigate the possibility that the copepods could derive nourishment from bacteria, three sets of tests were run in which the particulate matter was removed by passing the water through a Berkefeld filter (Table III). In the first set of tests less than half the *Calanus* in the Berkefeld-filtered water survived and in the other tests all succumbed. In the untreated water a definitely larger proportion of copepods was

alive at the end of one month. The addition of *Nitzschia* did not improve the survival, and in the first test it was attended by a very high mortality, although the reason for this is not known. Passing the water through a paper filter evidently did not remove material necessary for the copepods, since the survival under these circumstances was no poorer than in the case of the untreated water. Finally, when bacteria were added to the Berkefeld filtered water, the mortality was not as great as it was in the control experiment without the bacteria.

Although the results of these experiments seem to support the suggestion that the copepods were depending more on minute organisms than on the larger diatoms and green flagellates for food, their empirical nature renders them far from conclusive. The complete flora of the culture dishes is not known, nor were its changes followed during the course of the experiment. Furthermore, the length of time that *Calanus* can live without any food is in doubt because even the Berkefeld-filtered water was contaminated as soon as non-sterile copepods were placed in it.

Since no method for sterilizing copepods has yet been devised to our knowledge (cf. Bond, 1933), the interference from extraneous bacteria was minimized by causing fresh culture media to flow continuously through the flasks within which the copepods were confined—a scheme resorted to previously for a similar purpose (Gellis and Clarke, 1935). A continuous flow was provided by running the culture water through glass tubes from carboys placed on a shelf through submerged cooling coils to 500 cc. Erlenmeyer flasks set in the constant temperature tank (15° C.). Each flask was fitted with a two-hole rubber stopper through which inflow and outflow tubes passed. The end of the outflow tube within the flask was covered with netting of bolting silk and its level adjusted so that 400 cc. of liquid remained in the flask. The rate of flow was kept constant by arranging the carboys as "Mariotte's Bottles" (McCarthy, 1934) and the flow was suitably regulated by stopcocks to allow the entire contents of each carboy (22 liters) to pass through the flask connected with it in 24 hours.

In the first experiment sea water from the laboratory tap was passed through Whatman No. 12 filter paper and placed directly in Carboy *A*. The water for Carboy *B*, after being filtered through paper, was passed through the Berkefeld filter to remove the particulate matter. Although small quantities of water may be rendered absolutely sterile by Berkefeld filtering, complete removal of bacteria is not to be expected when large amounts of water are required. However, the existence of a significant difference in the numbers of bacteria present in the two containers was indicated by a bacterial count (using agar plates with suitable dilutions)

made 12 hours after the carboys had been filled. Carboy *A* was found to contain 680,000 bacteria/cc. and Carboy *B*, 120,000/cc.

Twenty specimens of *Calanus finmarchicus* were placed in each flask. After 20 days a total of 8 dead animals and 5 molted shells had been removed from Flask *A* (paper-filtered water). From Flask *B* (Berkefeld filtered water) 12 dead animals and no molted shells had been removed. In Flask *A* 11 copepods were still alive (one animal missing), whereas in Flask *B* only 2 copepods had survived (6 missing). It is clear, therefore, that in the Berkefeld-filtered water more animals were known to have died, many fewer were found still alive, and none had molted.

These results so strongly supported the idea that the copepods derived an important part of their nourishment from minute organisms that a second experiment using flowing water and designed to provide much greater differences in the numbers of bacteria present was immediately undertaken. To obtain a culture medium more nearly completely devoid of particulate matter, a more effective method of removing the bacteria was required. Resort was therefore made to a Zsigmondy ultra-filtration apparatus, using membrane filters of the "fine" type (average pore size about one micron to fifty millimicrons; said to remove "positively all germs and bacteria" and "the finer colloids"). Harbor water was collected from the Laboratory float and passed through Whatman No. 12 filter paper, then through the Berkefeld filter, and finally through the membrane filter. After this process, which required 6 hours or more, the water was placed in one of the three carboys used in this experiment (Carboy No. 3, Table IV). Into another of these carboys (Carboy No. 1) was put harbor water which had been passed through filter paper only and thus contained its original population of bacteria unaltered. An attempt was made to compare with this "normal" water not only water from which the bacteria had been removed but also water in which the number of bacteria was augmented. Dr. S. A. Waksman had found that if sea water was brought into the laboratory and left standing in the dark at room temperature, the number of bacteria increased enormously, reaching a maximum after 3 days. Accordingly, in the remaining carboy (Carboy No. 2) was placed harbor water which had been passed through filter paper and then allowed to stand for 3 days (or 2 days, see below).

A 24-hour supply of the 3 types of culture media was prepared every day, and just before use, cotton-filtered air was drawn through each for half an hour to insure the presence of sufficient oxygen. In this experiment two flasks were connected to each carboy; the tests were thus run in duplicate. The ends of both the inflow and the outflow of every

flask were covered by netting, thus precluding the possibility of the animals escaping up the tubes. As a control, a fourth pair of flasks was set up which was not provided with flowing water. The paper-filtered harbor water placed in these remained unchanged throughout the experiment. Each of the 8 flasks contained 20 *Calanus* in 300 cc. of water. All were examined every day and any dead animals and molted shells were removed.

At the end of 14 days it was found that the copepods in the flowing, paper-filtered water (Flasks 1*A* and *B*, Table IV) had fared the best, a total of 35 specimens being still alive, and 32 shells having been molted. The next best survival occurred in the controls (Flasks 4*A* and *B*), in which the water remained unchanged, with 33 copepods alive and 20

TABLE IV

The survival of *Calanus* in flowing water in relation to the abundance of bacteria. Twenty copepods per flask. Temperature: 12° C. Results at the end of 14 days. Roman numerals refer to the copepodid stages of *Calanus*.

Flask	Culture water	Total dead	Total molts	Alive
1*A*	Paper filtered	1 Cent.,* 1 Lab.	10 V, 5 IV	2♂, 9♀, 6 V =17
1*B*		1 Cent., 2 V	12 V, 5 IV	0♂, 12♀, 6 V =18
2*A*	Paper filtered, allowed to stand	1♂, 1 V	8 V, 1 IV	0♂, 8♀, 8 V, 1 IV =17
2*B*		1 Cent., 3 V, 1 IV	3 V, 3 IV	0♂, 4♀, 6 V, 1 IV =11
3*A*	Membrane filtered	8 V, 4 IV	0	0♂, 2♀, 5 V = 7
3*B*		1 ♀, 13 V, 1 IV	0	0 = 0
4*A*	Paper filtered, water unchanged	1 Cent., 1 V	5 V, 4 IV	0♂, 8♀, 9 V =17
4*B*		3 V	7 V, 4 IV	2♂, 9♀, 5 V =16

* Four *Centropages* and one *Labidocera* were found to have been introduced by mistake. They were all among the first to die.

molted shells. In the paper-filtered water which had been allowed to stand (Flasks 2*A* and *B*) 28 animals were alive and 15 molts had occurred. Survival in the membrane-filtered water (Flasks 3*A* and *B*) was decidedly inferior, only 7 animals being alive and no molts occurring. In five cases the dead and living animals totaled only 19 and in one case they totaled 21. These discrepancies may be due to errors in counting when the copepods were first placed in the flasks. But 4 animals were missing from Flask 2*B* and 5 animals from Flask 3*B*, and in the previous experiment 6 animals from Flask *B* were unaccounted for. Since in the last two cases the culture water was supposedly deficient in food, the possibility suggests itself that the missing animals were devoured by the others (cf. Lebour, 1922). On one occasion a copepod

which had been alive on the previous day was found in a half demolished condition.

Through the kind assistance of Dr. C. L. Carey it was possible to make bacterial counts of the culture media at three times during the course of this experiment. On the first day after the beginning of the experiment the following numbers of bacteria were found:

Flask 1*A*	580,000	bacteria/cc.
Flask 2*A*	130,000	"
Flask 3*A*	6,000	"
Carboy 3	140	"
Flask 4*A*	210,000	"

This count showed that a very pronounced difference existed in the sizes of the bacterial populations in Flasks 1*A* and 3*A*, but contrary to expectation there were fewer bacteria in Flask 2*A* than in Flask 1*A*. A more elaborate count was therefore made on the fifth day:

Carboy	Days after collecting	Bacteria per cc.	Flask	Bacteria
1	0	6,000	1*A*	88,000
2	4	9,000	2*A*	28,000
2*a*	3	1,000		
2*b*	2	45,000		
2*c*	1	14,000		
3	1	8,000	3*A*	220,000

The maximum number of bacteria in the standing carboys was evidently reached after 2 days instead of after 3 days. The procedure of the experiment was altered accordingly, and beginning on the seventh day, Carboy No. 2 was allowed to stand only 2 days. Furthermore, the populations in Flasks 1*A* and 2*A* are very much smaller than they were on the first day, and the population in Flask 3*A* has become enormously increased. In all three flasks the numbers of bacteria were much greater than in the corresponding carboys which were supplying them. The explanation for this is not known. Evidently the flora is subject to violent and rapid fluctuations, and in any future attempt to study the precise relationships between bacteria and copepods it will be necessary to follow these changes much more closely than was possible in the present case.

The extremely large number of bacteria found on this occasion in Flask 3*A*, which was supposed to be devoid of particulate matter, aroused the suspicion that the filtering process was faulty. The following counts were therefore made on the seventh day.

Carboy 3	immediately after filtering	9	bacteria/cc.
" "	" aërating	7	"
" "	17 hours after starting flow (1 cc. direct)	88	"
" " "	" " starting flow (10–1 dil.)	530	"
Flask 3*A* "	" " starting flow (10–1 dil.)	890	"

These results indicate that the use of membrane filters is as effective in removing the bacteria as could be expected for such large volumes of water. In addition, it seems probable that the large population apparently present in Flask 3*A* on the fifth day was exceptional, and that taking the whole period of the experiment, the copepods in this flask were provided with a decidedly smaller number of bacteria than those in the other flasks.

There is no question but that the copepods in Flasks 1*A* and *B* were thriving throughout the course of the experiment and that those in Flasks 3*A* and *B* were suffering adverse conditions. Little can be concluded regarding the copepods in Flasks 2*A* and *B* because of the uncertainty as to the numbers of bacteria present. It may be significant, however, that all but two of the molts which occurred in these flasks took place after the seventh day—when the period for which the water was allowed to stand was changed from 3 days to 2 days. Good survival and a relatively large number of molts were found in the control flasks (4*A* and *B*) although 40 animals would have been expected to have consumed all the food material present in a very few days. The bacteria originally in the culture water undoubtedly could multiply faster than the copepods would eat them provided that sufficient nutrient substances for the bacteria were present. Although in plain sea water the nutrients would be largely exhausted in three or four days, it is possible that the material excreted by the copepods served as a supplementary source of nourishment for the bacteria and permitted a sizable flora to maintain itself for the fourteen days of the experiment.

If the foregoing interpretation is accepted, the experiment appears definitely to point to the dependence of the *Calanus* on bacteria or other microörganisms. But a possible alternative explanation of the whole experiment should be considered, namely, that none of the copepods were feeding to any significant extent and that the differences in the three tests were due to other factors than the nutritional. Several investigators have suggested that at certain times under natural conditions copepods may live for long periods of time with a greatly reduced diet (cf. Campbell, 1934; Marshall, 1924). In the present experiment the elaborate filtering process used for Flasks 3*A* and *B* might conceivably have rendered the culture water harmful in some respect, and the period of standing employed in the case of the water for Flasks 2*A* and *B* might have allowed lethal bacteria to multiply unduly. The

slight improvement of Flasks 1*A* and *B* over Flasks 4*A* and *B* could be explained on the basis of a better supply of oxygen and removal of metabolites in the flasks with flowing water. However, the difference between these two sets of flasks is very slight in respect to survival, but in number of molts, the former set is superior by 50 per cent; and in the other cases longer survival is correlated with a greater amount of molting. Although it is possible that stored oil could be entirely depended upon for the production of new shells, the growth entailed in the process would seem to presuppose the taking in of a considerable quantity of nutriment.

Everything considered, the interpretation of the experiment on the nutritional basis seems the more probable one. According to this, the copepods were living and growing on bacteria or other food material small enough to pass through the pores of the paper filter, and when this particulate matter was removed, the animals failed to grow and died off rapidly.

Discussion

Since the time of Lohmann it has been known that the nannoplankton constitutes a large *potential* source of food in the sea, but little has been done to show whether this is actually used by the zoöplankton (cf. Bond, 1933). The foregoing experiments are by no means conclusive, but if, as they indicate, copepods in nature feed to any significant extent upon material as minute as bacteria, a knowledge of the precise organisms and relationships involved will be necessary for an understanding of this part of the food-cycle of the sea. Besides the bacteria, of which there are many types in the sea, other kinds of particulate matter —both living and non-living—should be scrutinized as possible food sources. A fresh-water entomostracan, *Daphnia*, has been shown to be able to derive a certain amount of nourishment from material present in colloidal form (Gellis and Clarke, 1935). Colloidal material undoubtedly exists in sea water, but little or nothing is known of its abundance or availability. Dr. J. B. Lackey, who was conducting a survey of the Protista of Woods Hole Harbor at the time that these experiments on *Calanus* were in progress, has very kindly informed me that of the organisms, other than bacteria, present in the harbor water which would pass in any significant number through Whatman No. 12 filter paper, the following were the most numerous: the spermatozoa of fish and of invertebrates, the gametes or swarm spores of algae, flagellate Protozoa, and unidentified algal cells 2–5 μ in diameter. Of these the flagellate Protozoa would constitute the largest bulk of food material.

The majority of the diatoms—at least the larger ones—was presumably removed by the filter paper; but diatoms and all other larger organisms may be important as a food source when broken into fragments during the course of their disintegration after death. Furthermore, after the bodies of dead plants and animals have passed finally into solution, their substance serves as nutriment for countless bacteria. Larger organisms may thus indirectly furnish nourishment for the copepods. The excretions of living organisms possibly also support a useful population of bacteria. There is some evidence that part of the material synthesized by diatoms dissolves out of the living cells into the surrounding water (Pütter, 1924; Gran, 1927; Marshall and Orr, 1930; and Krogh, Lange, and Smith, 1930). Waksman et al. (1933) found that in the Gulf of Maine many more bacteria existed in association with the zoöplankton and the phytoplankton than in the free water. They state:

"A decided parallelism was observed between the abundance of diatoms in the sea and abundance of bacteria. . . . These results seem to point definitely to the fact that the development of phytoplankton in the sea is accompanied closely by bacterial development. The bacteria feed upon the excretion products of the diatoms, algae, and animal forms and probably upon these plankton forms themselves as soon as they die. . . ." The explanation of those cases in which a correlation is claimed between the abundance of copepods and of diatoms may possibly be that the copepods depend for their nourishment upon the diatoms *indirectly* through the bacteria.

Summary

1. The importance of copepods in the economy of the sea and the inconclusive and contradictory nature of previous observations on their food make it desirable to investigate the nutrition of these animals in a thorough manner. For this purpose and in order to develop a satisfactory method of culturing copepods in the laboratory so that their physiology might be studied under carefully controlled conditions, feeding experiments were undertaken using *Centropages, Labidocera, Acartia,* and particularly *Calanus.*

2. Small containers without stirrers appeared to be suitable provided that over-crowding was avoided and a low temperature maintained. The materials which were tested as possible sources of food included the organisms present in the harbor water and "persistent" cultures of certain diatoms and green flagellates.

3. Although the survival of the first three copepods mentioned was prolonged by the addition of the diatoms and green flagellates, the

majority died off after about two weeks. In the case of *Calanus* only a slight improvement resulted from this treatment and a large proportion survived when no food organisms were added.

4. Further experiments revealed that the sterilization of the culture water and the removal of particulate matter from it was accompanied by a high mortality of *Calanus* and that the addition of bacteria to the water resulted in improved survival. To test the possibility that the copepods were utilizing the smaller types of microörganisms for food, the growth of bacteria, etc. was minimized by passing membrane-filtered water continuously through flasks in which certain of the copepods were confined. These copepods failed to molt and died off rapidly, whereas those in flasks through which paper-filtered water flowed remained alive and molted in large numbers.

5. These experiments therefore indicate that bacteria and other constituents of the nannoplankton may be an important food for copepods in the sea. Diatoms and other larger organisms may possibly serve as a source of nourishment indirectly through the bacteria, etc., which feed upon them and their excretions.

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BIBLIOGRAPHY

ALLEN, E. J., AND E. W. NELSON, 1910. On the Artificial Culture of Marine Plankton Organisms. *Jour. Mar. Biol. Ass.*, N.S., **8** (No. 5) : 421.

BIGELOW, H. B., 1926. Plankton of the Offshore Waters of the Gulf of Maine. *Bull. U. S. Bur. Fish.*, **40:** 1.

BOND, R. M., 1933. A Contribution to the Study of the Natural Food-cycle in Aquatic Environments with Particular Consideration of Microörganisms and Dissolved Organic Matter. *Bulletin Bingham Oceanographic Collection*, **4** (Art. 4) : 1.

BOND, R. M., 1934. Digestive Enzymes of the Pelagic Copepod, Calanus finmarchicus. *Biol. Bull.*, **67:** 461.

CAMPBELL, M. H., 1934. Calanus tonsus (Brady) as an Economic Factor in the Strait of Georgia. *Fifth Pacific Science Congress, Vancouver, B. C.*, A5.3., pp. 2003–2008.

CANNON, H. G., 1928. On the Feeding Mechanism of the Copepods, C. finmarchicus and Diaptomus gracilis. *Brit. Jour. Exper. Biol.*, **6:** 131.

CLARKE, G. L., 1934*a*. The Rôle of Copepods in the Economy of the Sea. *Fifth Pacific Science Congress, Vancouver, B. C.*, A5.5., pp. 2017–2021.

CLARKE, G. L., 1934*b*. Factors Affecting the Vertical Distribution of Copepods. *Ecological Monographs*, **4:** 530.

CRAWSHAY, L. R., 1915. Notes on Experiments in the Keeping of Plankton Animals under Artificial Conditions. *Jour. Mar. Biol. Ass.*, N.S., **10:** 555.

DAKIN, W. J., 1908. Notes on the Alimentary Canal and Food of the Copepods. *Intern. Rev. Hydrobiol. u. Hydrogr.*, **1**: 772.

ESTERLEY, C. O., 1916. The Feeding Habits and Food of Pelagic Copepods and the Question of Nutrition by Organic Substances in Solution in the Water. *Univ. Calif. Publ. Zool.*, **16**: 171.

FISH, CHARLES J., 1925. Seasonal Distribution of the Plankton of the Woods Hole Region. *Bull. Bur. Fish.*, **41**: 91.

GELLIS, S. S., AND G. L. CLARKE, 1935. Organic Matter in Dissolved and in Colloidal Form as Food for Daphnia magna. *Physiol. Zool.* (in press).

GRAN, H. H., 1927. The Production of Plankton in the Coastal Waters off Bergen, March–April 1922. *Rept. Norweg. Fish. and Mar. Investigation*, **3** (8).

HAGMEIER, A., 1930. Die Züchtung verschiedener wirbelloser Meerestiere. 1930. *Abderhalden: Handb. biol. Arbeit.*, Abt. 9, Teil 5, Heft 4, Lief. 326, pp. 465–598.

HARDY, A. C., 1924. The Herring in Relation to its Animate Environment. Part I. The food and feeding habits of the herring with special reference to the east coast of England. *Min. Agric. Fish., Fish. Invest.*, Ser. 2, **7**: 1.

HARVEY, H. W., 1928. Biological Chemistry and Physics of Sea Water. Cambridge University Press. Cambridge, England.

HJORT, J., AND T. T. RUUD, 1929. Whaling and Fishing in the North Atlantic. *Rapp. Proc. Verb. Cons. Inter. Explor. Mer.*, **56**: 1.

JOHNSTONE, J., 1911. Life in the Sea. Cambridge University Press. Cambridge, England.

KROGH, A., E. LANGE, AND W. SMITH, 1930. On the Organic Matter Given Off by Algae. *Biochem. Jour.*, **24**: 1666.

LEBOUR, M. V., 1922. The Food of Plankton Organisms. I. *Jour. Mar. Biol. Ass.*, **12**: 644.

MARSHALL, SHEINA, 1924. The Food of Calanus finmarchicus during 1923. *Jour. Mar. Biol. Ass.*, **13**: 473.

MARSHALL, S. M., A. G. NICHOLLS, AND A. P. ORR, 1934. On the Biology of Calanus finmarchicus. V. Seasonal distribution, size, weight, and chemical composition in Loch Striven in 1933, and their relation to the phytoplankton. *Jour. Mar. Biol. Ass.*, **19**: 793.

MARSHALL, S. M., AND A. P. ORR, 1930. A Study of the Spring Diatom Increase in Loch Striven. *Jour. Mar. Biol. Ass.*, **16**: 853.

MCCARTHY, E. L., 1934. Mariotte's Bottle. Science, **80**: 100.

MURPHY, HELEN E., 1923. Life Cycle of Oithona nana, Reared Experimentally. *Univ. Calif. Publ. Zool.*, **22**: 449.

PÜTTER, A., 1924. Der Umfang der Kohlensaurereduktion durch die Planktonalgen. *Pflügers Arch. Physiol.*, **205**: 293.

SAVAGE, R. E., 1931. The Relation between the Feeding of the Herring off the East Coast of England and the Plankton of the Surrounding Waters. *Min. of Agric. and Fish., Fish. Invest.*, Ser. 2, **12**: 4.

WAKSMAN, S. A., H. W. REUSZER, C. L. CAREY, M. HOTCHKISS, AND C. E. RENN, 1933. Studies on the Biology and Chemistry of the Gulf of Maine. 3. Bacteriological investigations of the sea water and marine bottoms. *Biol. Bull.*, **64**: 183.

WIMPENNY, R. S., 1929. Preliminary Observations on the Fat-content of the Plankton on the English Herring Grounds in the North Sea. *Min. of Agric. and Fish., Fish. Invest.*, Ser. 2, **11**: 5.

YONGE, C. M., 1931. Digestive Processes in Marine Invertebrates and Fishes. *Jour. du Conseil*, **6**: 175.

FURTHER EVIDENCE OF A DIURNAL RHYTHM IN THE MOVEMENT OF PIGMENT CELLS IN EYES OF CRUSTACEANS

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Diurnal rhythms which persist under constant environmental conditions have long interested biologists and although numerous instances of such are now known we still lack definite information regarding the internal processes which are involved in such rhythms. Among the many daily changes in activities of animals, some of which have been previously mentioned by the author (Welsh, 1930), the movements of the pigment cells in arthropod eyes would appear to be quite satisfactory for studying the physiological mechanism underlying persisting diurnal rhythms. Kiesel (1894) and Demoll (1911) were the first to observe a daily rhythm in the "glow" of the eyes of certain moths when these were kept in constant darkness. Welsh (1930) first reported a daily rhythm in the crustacean eye. This was in two species of *Macrobrachium,* fresh water shrimp, which were studied in Cuba. In these forms, when kept under constant illumination, the distal pigment cells of the eye assume the position characteristic for the dark at the time of sunset and do not return to the light position until sunrise the following morning. A daily variation in the position of the proximal pigment of the eye of *Cambarus,* when this form is kept in constant darkness, has been reported by Bennitt (1932). Various suggestions have been offered to explain the persistence of such rhythms but none of them are supported by adequate experimental data. The rich crustacean fauna of Bermuda should offer material for such studies and the purpose of the present paper is to show that persisting diurnal rhythms do exist in several Bermuda shrimp and prawn eyes and in all of the three sets of pigment cells found in the eyes of such forms.

If daily variations in the position of pigment cells in the eye occur, and if they continue under constant environmental conditions, they may be best detected by keeping animals in constant light or darkness and killing and fixing some during daylight hours and others during the night. Subsequent comparison of sections of such eyes would then show the condition of both light-adapted and dark-adapted eyes as they appear during the day or night. Such a procedure was

used in a search for rhythms in eye pigment in Bermuda shrimp and prawn and the condition in the eyes of four species, representative of those studied, follows. The species to be described were selected as representing three families and at the same time including species which are active only at night and others which are most active during the daytime. They are all forms which are easily obtained in abundance and therefore satisfactory for experimental work.

In preparing material for study, animals were light-adapted or dark-adapted for several hours and then fixed in hot water, some during the day and others at night. The head bearing the eyes was then cut off and placed in 70 per cent alcohol. At least three eyes fixed under each of the four conditions were then sectioned and studied and the typical condition is shown in Plate I. The single ommatidia are scale drawings adapted from camera lucida sketches and in each ommatidium the positions of the distal, proximal, and accessory pigments are shown. For general information regarding the movement of retinal pigment one may refer to the excellent review by Parker (1932). It may be well to mention here, however, that all three sets of pigment are involved in controlling the amount of light which reaches the rhabdomes or image receptors. Typically, in the light condition during the day, the distal pigment cells rest on top of the retinular cells, the proximal pigment surrounds the rhabdomes, and the accessory or reflecting pigment is beneath the basement membrane. Fig. *B* in each series shows this condition. The normal dark condition as seen at night is shown in Fig. *C* of each series. In this condition the distal pigment cells move out along the cones, the proximal pigment migrates below the basement membrane, and the reflecting (white) pigment moves up to form a screen back of the rhabdomes. This situation permits the maximum amount of light to enter the sensitive elements.

Any variation in the position of the pigment in eyes of light-adapted animals fixed at night (Fig. *A*) or of dark-adapted animals killed during the daytime (Fig. *D*) would indicate some internal control other than that resulting from the direct stimulation of the eye by light or

Explanation of Plate I

Series 1. Ommatidia of eyes of *Latreutes fucorum* (× 100) showing positions of distal, proximal, and reflecting pigment of (*A*) light-adapted animals killed at night; (*B*) light-adapted animals killed during day; (*C*) dark-adapted animals killed during night; (*D*) dark-adapted animals killed during day.

Series 2. Ommatidia of eyes of *Leander tenuicornis* as in Series 1, (× 50).

Series 3. Ommatidia of eyes of *Leander affinis* as in Series 1, (× 50).

Series 4. Ommatidia of *Penaeopsis goodei* as in Series 1, (× 50).

D = distal pigment.
P = proximal pigment.
R = reflecting pigment.

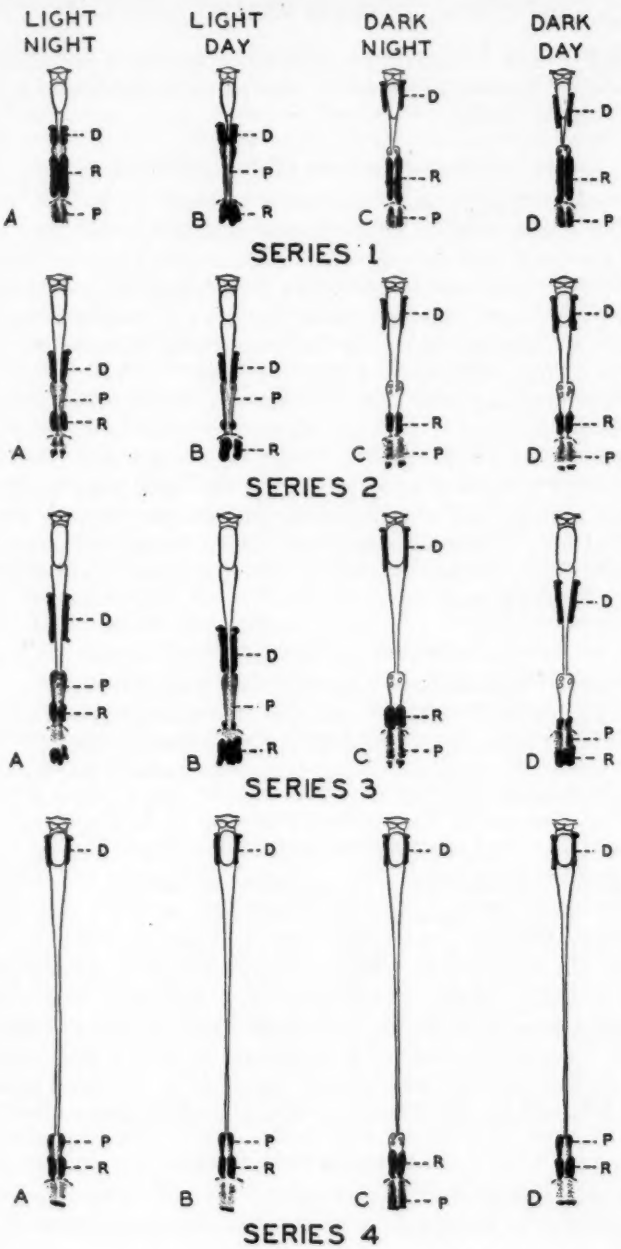
LIGHT NIGHT
LIGHT DAY
DARK NIGHT
DARK DAY
A
B
C
D
D
P
R
SERIES 1
SERIES 2
SERIES 3
SERIES 4

PLATE I

the lack of such a stimulus. In each series some such variations may be seen and these variations will be pointed out in the consideration of each separate species.

Latreutes fucorum (Fabr.) Stebbing

Family: Hippolytidae. "Gulf-weed Shrimp."

This species is found on Sargassum weed in large numbers where it is associated with the other very characteristic fauna of the gulf-weed. It is well protected by its color resemblance to the weed and its tendency to remain quiescent during the day. Its exposure to fairly brilliant illumination during the day necessitates a mechanism in the eye to care for a wide range of light intensities.

When the eyes of this form are studied, the most striking rhythm is seen in the reflecting or accessory pigment (Figs. *A–D*, Series 1). If we compare the ommatidia from "night-light" eyes with those from "day-light" eyes, it is seen that during the night, even though the animals are subjected to illumination, the reflecting pigment still remains above the basement membrane. Slight variations from normal dark and light conditions may also be seen in the positions of the distal and proximal pigment.

Leander tenuicornis (Say)

Family: Palaemonidae. "Common Gulf-weed Shrimp."

The habits and habitat of this form are like those of *Latreutes*. Similarly the most striking rhythm is in the reflecting pigment (Figs. *A–D*, Series 2). This is of some interest as *Leander* and *Latreutes* belong to different families.

Leander affinis (Milne-Edwards)

Family: Palaemonidae. "Transparent Shrimp."

This species of *Leander* differs considerably from *L. tenuicornis* in its habits. It is active chiefly during the daytime, when it is found in schools close to the shore. It is well protected by its relative transparency, which renders it difficult to see. Light and dark-adapted ommatidia from eyes fixed during the day and during the night are seen in Figs. *A–D*, Series 3. A definite rhythm in the movements of the reflecting pigment and a partial migration of the distal pigment occur daily whether the animals are kept in constant light or darkness.

Penaeopsis goodei (Smith)

Family: Penaeidae.

The habits of this species of prawn were probably observed for the

first time while this study was being made. They are of sufficient interest to recount briefly. *P. goodei* is a distinctly nocturnal form found swimming at the surface in the late evening between 8:30 and 10:30 P.M. It can easily be seen by the brilliant reflection of light from the eyes when a search-light is played over the surface of the water. During the day this prawn is found buried in the sand. Specimens were obtained on two occasions when dredging for other sand-dwelling forms.

Besides a very definite daily periodicity in activity this form is also found swimming at the surface in largest numbers at the dark of the moon, so that it may be said to exhibit a lunar periodicity as well. The observations made during the course of this work have since been further substantiated by observations made by Dr. J. F. G. Wheeler, Director of the Bermuda Station.

When the eyes of *Penaeopsis* are examined (Figs. *A–D*, Series 4), it is seen that the distal pigment cells fail to show any movement. They remain in a fixed position around the distal portion of the cones. Likewise the reflecting pigment maintains the same position in the light or in the dark. The only movement which occurs is in the proximal pigment, which migrates below the basement membrane when animals are dark-adapted during the night. During the daytime, however, even though the animals are kept in the dark the proximal pigment returns to the retinular cells, which is the normal light position. The only rhythm in this form, therefore, is that of the proximal pigment, when animals are kept in constant darkness. In this respect *Penaeopsis* resembles *Cambarus*.

It is evident from this and earlier studies that diurnal rhythms involving changes in eye pigment are widespread among such crustaceans as shrimp and prawn. It now remains to be shown what underlying physiological rhythms are involved. The most recent evidence which promises to aid in an understanding of this phenomenon in crustacean eyes is that of Kleinholz (1934), who has shown that the eye-stalk hormone, first found by Perkins (1928) and Koller (1928) to affect the body chromatophores, also plays a part in the movement of the distal pigment cells. Kleinholz has demonstrated that an extract of the eye-stalks of light-adapted animals when injected into animals which have been kept in the dark causes a complete migration of the distal pigment cells to the position characteristic of the light. More recent unpublished results indicate that the eye-stalk hormone has no effect on the proximal or reflecting pigments. This is the first convincing demonstration of hormone effects on crustacean eye pigments and also furnishes additional evidence indicating that the different pig-

ments of the eye are under different controls. Apparently it is possible to obtain by means of the eye-stalk hormone a situation opposite to that in the eyes of *Macrobrachium* (Welsh, 1930), where by keeping the animals under constant illumination the distal pigment, at night, assumes the position characteristic of the dark, while the proximal pigment remains in the light condition.

If diurnal rhythms under constant conditions occurred only in the movements of the distal pigment cells, we would seem to be approaching an explanation of the underlying process, but the fact that such rhythms also occur in the movements of the proximal and reflecting pigments only makes a complicated situation more difficult to understand.

In conclusion the author wishes to acknowledge his indebtedness to Doctors G. L. Clarke and F. A. Brown, who collected in Bermuda some of the material used in this study, to Dr. F. A. Chace, Jr., who did the identifications, and to Miss Virginia Macdonald, who assisted in the histological preparation of the material.

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LITERATURE CITED

BENNITT, R., 1932. Diurnal Rhythm in the Proximal Pigment Cells of the Crayfish Retina. *Physiol. Zool.*, **5**: 65.

DEMOLL, R., 1911. Über die Wanderung des Irispigments im Facettenauge. *Zool. Jahrb., Abt. allg. Zool. u. Physiol.*, **30**: 169.

KIESEL, A., 1894. Untersuchungen zur Physiologie des facettirten Auges. *Sitzungsber. Akad. Wiss., Wien.*, Abt. 3, **103**: 97.

KLEINHOLZ, L. H., 1934. Eye-stalk Hormone and the Movement of Distal Retinal Pigment in Palaemonetes. *Proc. Nat. Acad. Sci.*, **20**: 659.

KOLLER, G., 1928. Versuche über die inkretorischen Vorgänge beim Garneelenfarbwechsel. *Zeitschr. vergl. Physiol.*, **8**: 601.

PARKER, G. H., 1932. The Movements of the Retinal Pigment. *Ergebnisse der Biologie*, **9**: 239.

PERKINS, E. B., 1928. Color Changes in Crustaceans, Especially in Palaemonetes. *Jour. Exper. Zool.*, **50**: 71.

WELSH, J. H., 1930. Diurnal Rhythm of the Distal Pigment Cells in the Eyes of Certain Crustaceans. *Proc. Nat. Acad. Sci.*, **16**: 386.

A CORRELATION BETWEEN THE FOOD EGGS OF FASCIOLARIA TULIPA AND THE APYRENE SPERMATOZOA OF PROSOBRANCH MOLLUSCS

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It has long been known in *Fasciolaria tulipa* that of at least 600–800 eggs laid in a capsule only a few develop. Those eggs that fail to develop are eaten by the normal larvæ. Glaser (1905), in a study of this "egg cannibalism" in *Fasciolaria,* has suggested that these food eggs represent a condition comparable to that found in oligopyrene sperm, such as Meves (1901) described for *Paludina.* During a study of Professor E. G. Conklin's preparations of *Fasciolaria,* we have found cytological evidence that permits an elaboration of Glaser's suggestion. Moreover, because of the more recent work of Reinke (1912, 1914) on the apyrene sperm of *Strombus,* it is possible to make a more adequate correlation between atypical eggs and spermatozoa.

The material examined consists of eggs prepared by Professor E. G. Conklin in 1903 at Beaufort, N. C. In addition to a series of untreated eggs, several series were treated with the following parthenogenetic reagents:

1 per cent NaCl for 1½ hours
2 per cent NaCl for 12 hours
4 per cent $MgCl_2$ for 10 hours
8 per cent $MgCl_2$ for 12 hours
1 drop of HCl in 300 cc. sea water.

We are indebted to Professor E. G. Conklin for his kindness in putting his preparations at our disposal and for suggesting this study.

In cytological detail the food eggs of *Fasciolaria* are more comparable to apyrene sperm than to oligopyrene ones. Meves (loc. cit.) described a type of spermatocyte in *Paludina* which had abnormal maturation divisions, wherein the chromosomes were irregularly distributed to the resulting cells; further the spermatids transformed abnormally. These spermatozoa were designated "oligopyrene sperm." In 1912 Reinke, working with *Strombus,* found that the primary atypical spermatocytes did not undergo any maturation division but transformed directly into abnormal spermatozoa. Their nuclei fragment into coarse

chromatin granules, which become vesiculate, fade, and disappear. This type of sperm is called "apyrene."

The food eggs of *Fasciolaria* also have no maturation divisions. When laid they have a large, faintly reticulate germinal vesicle in which there is an abnormal giant nucleolus (Fig. 1). Usually the nucleus remains in this condition until degeneration occurs. Glaser (1907) has described this degeneration as an "explosive" process, whereby the nucleus is broken into vesicles containing bits of the nucleolus. He failed to find evidences of amitosis such as Osborn (1904) described. We have been able to find stages intermediate between the germinal vesicle and the fragments observed by Glaser; for frequently the nucleolus and the nuclear membrane are distorted (Fig. 2), suggesting that this distortion will lead to fragmentation in a somewhat slower manner than that described by Glaser, although the result is the same.

In other eggs there are feeble attempts at a maturation division. In some of these eggs the fine chromatin reticulum of the germinal vesicle becomes condensed into imperfectly formed chromosomes. These chromosomes are grouped together within the nuclear membrane; the membrane itself may be distorted so that the chromosomes are somewhat separated from the nucleolus (Fig. 3). On the other hand, the chromosomes may form more perfectly and arrange themselves in the semblance of a mitotic figure, in which no spindle fibers are to be seen (Figs. 4 and 5). Here the nucleolus may or may not disappear or become separated from the chromosomes.

The attempt at division goes no farther than this. The nucleus and chromosomes undergo degeneration in much the same manner as described for the chromatin granules of the atypical spermatocytes of *Strombus*. Vacuolate chromosomal vesicles appear, with the deeply staining chromatin lining their walls. These vesicles enlarge and their contents stain less and less deeply. During this process the nucleolus may or may not fragment. In many cases the nucleolus can be seen to break into several faintly staining bodies which become scattered in the cytoplasm; but in other instances the nucleolus does not fragment, but becomes very faintly stained and is surrounded by a ring of degenerating vesicles (Fig. 6). The treatment with parthenogenetic reagents did not cause development of the eggs. The important fact is, as Osborn, Glaser, and we have found, that normally there are no maturation divisions in the food eggs of *Fasciolaria*. This fact presents a situation comparable with the apyrene sperm of *Strombus*, and other forms. That all the eggs are not arrested in the same stage presents a correlation with the spermatozoa of this species; for Hyman (1923) has described three types of atypical spermatozoa.

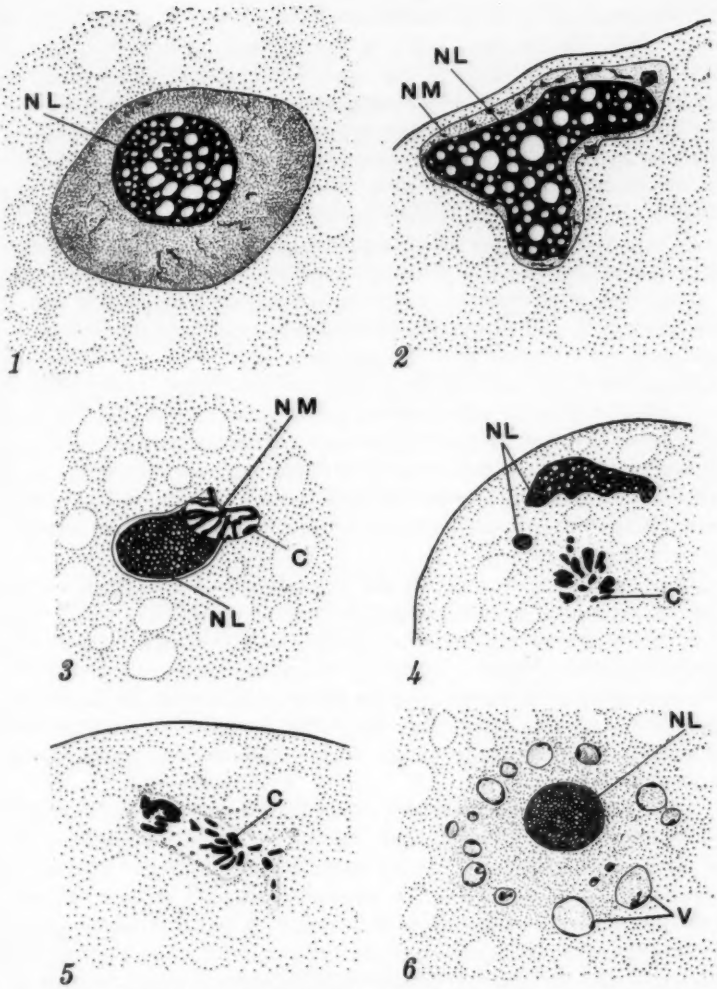

FIG. 1. Germinal vesicle before fragmentation. Nucleolus, (*nl*) large and vacuolate. Yolk represented by blank areas.

FIG. 2. Lobulate nucleus with nucleolus extending nearly to nuclear membrane (*nm*).

FIG. 3. Chromosomes (*c*) condensed and collected at one pole of the nucleus. Nuclear membrane still intact.

FIG. 4. Polar view of the chromosomes. Nucleolus undergoing fragmentation.

FIG. 5. Chromosomes arranged in a semblance of a mitotic figure. Nucleolus has disappeared.

FIG. 6. Chromosomal vesicles (*v*) forming a ring around the nucleolus. Degenerating stage.

Bütschli (1877) described irregular cleavages in *Paludina* eggs, in which there was an early arrest of development. Similarly *Buccinum* and *Purpura* show irregular cleavages, but in these cases maturation divisions occur. Hence, it is suggested that these atypical eggs of *Paludina, Buccinum,* and *Purpura* are comparable to oligopyrene sperm, whereas in *Fasciolaria* they are comparable to apyrene sperm. In the limited material of *Buccinum* at our disposal we have found abnormally large polar bodies, and in the cleavage cells, degeneration of chromatin.

The significant fact which arises from this study is that in the gametogenesis of both sexes of prosobranch molluscs, there exists among the various species a transition from the normal condition to one that is highly atypical. The least atypical deviation from the normal spermatogenesis is the *Paludina* type (oligopyrene), where there are maturation divisions but these are irregular. In the *Strombus* type (apyrene) there is a situation which is more accentuated than that of *Paludina,* for here both maturation divisions are suppressed. A similar transition is found in oögenesis. In *Buccinum* and other forms there are oligopyrene eggs wherein the deviation from the normal is less marked than in *Fasciolaria.* In the latter the maturation divisions are suppressed so that only vestiges of the process remain.

It is interesting to note that in *Fasciolaria* Hyman (1923) found that the ratio of atypical spermatozoa to normal was 50:1, which ratio is of about the same order of magnitude as that found between the atypical and typical eggs. Hyman, however, failed to realize that there are atypical eggs in *Fasciolaria;* but he considered that the failure of most of the eggs to develop was due to the fact that the great number of atypical spermatozoa produced did not leave enough typical sperm to fertilize them.

Since this paper was submitted for publication, a paper has appeared by Woodard (1935) on the apyrene spermatozoa of *Goniobasis laqueata.* In the formation of these atypical spermatozoa an abortive attempt at maturation is made, wherein "a variable number of bivalent chromosomes are formed in the nucleus by aberrant prophase processes; these are brought upon an abortive spindle, then scattered to all parts of the cell where they undergo progressive vacuolization." This condition in *Goniobasis* is closely paralleled in *Fasciolaria* (cf. Figs. 3–6), and presents another link in the evidence for the apyrene egg.

LITERATURE CITED

BÜTSCHLI, VON O., 1877. Entwicklungsgeschichtliche Beiträge. *Zeitschr. f. wiss. Zool.,* **29**: 216.

GLASER, O. C., 1905. Über den Kannabalismus bei Fasciolaria tulipa (var. dis-

tans) und deren larvale Excretionsorgane. *Zeitschr. f. wiss. Zool.*, **80**: 80.

GLASER, O. C., 1907. Pathological Amitosis in the Food-Ova of Fasciolaria. *Biol. Bull.*, **13**: 1.

HYMAN, O. W., 1923. Spermic Dimorphism in Fasciolaria tulipa. *Jour. Morph.*, **37**: 307.

MEVES, F., 1901. Über die sog. wurmförmigen Samenföden von Paludina und über ihre Entwicklung. Separat-Abdruck aus Mittheilungen für den Verein Schlesw.-Hölst. Aertze." Jahrgang X, nr. 1, 1901, s. 1–11.

OSBORN, H. L., 1904. Amitosis in the Embryo of Fasciolaria. *Am. Nat.*, **38**: 869.

REINKE, E. E., 1912. A Preliminary Account of the Development of the Ayprene Spermatozoa in Strombus and of the Nurse Cells in Littorina. *Biol. Bull.*, **22**: 319.

REINKE, E. E., 1914. The Development of the Apyrene Spermatozoa of Strombus bituberculatus. Marine Biological Laboratory at Tortugas. v. 6, pp. 197–239.

WOODWARD, T. M., 1935. Spermic Dimorphism in Goniobasis laqueata (Say). *Jour. Morph.*, **57**: 1.

A BUFFERED AND LOW OXYGEN CONTENT PHYSIOLOGICAL SALT SOLUTION

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A physiological salt mixture in which *Trichonympha campanula*, a protozoan symbiont of *Zootermopsis angusticollis*, could be kept alive was worked out by Cleveland (1925*a*). Protozoa are known to be extremely sensitive to osmotic pressure. The cell walls disrupt either inward or outward according as the osmotic pressure of the medium is larger or smaller than that of the fluid within the cell. The solution which Cleveland found most suitable was a salt mixture of approximate pH 7.2 and with an osmotic pressure equivalent to that of an approximately 0.40 per cent sodium chloride solution. This mixture was suitable for the Protozoa of many termite species, but the intestinal Protozoa of *Reticulitermes hesperus* could be kept alive in it for only a short length of time. Since the completion of our work, Trager (1934) has reported a salt mixture most suitable for the Protozoa of *Zootermopsis angusticollis* as having an osmotic pressure equivalent to that of a 0.3 to 0.4 per cent sodium chloride solution, and a pH range of 6.8 to 7.2.

Oxygen gas is used under pressure for the defaunation of termites (Cleveland, 1925*b*, *c*, *d*, 1928; Andrew, 1930). The oxygen thus forced into solution in the intestinal fluid is evidently an agent toxic to the Protozoa. This led to the suspicion that some of the intestinal Protozoa of *Reticulitermes hesperus* were unusually sensitive to dissolved oxygen and were affected by the small amount dissolved in Cleveland's solution. Trager (1934) has also later shown that some of the Protozoa of *Zootermopsis angusticollis* are very sensitive to oxygen. These grow best where air is excluded by vaseline seals or where the culture tubes connected by paraffined stoppers and glass tubing to other tubes contain alkaline pyrogallol thereby creating an atmosphere of low oxygen content. The amount of oxygen dissolved from the air at atmospheric pressure by water (Winkler, 1889) is small, being 8.8 ml. of oxygen per 1,000 ml. of water at 5.2° C., 7.1 at 14.8° C. and 5.8 at 24.8° C.

A desirable physiological salt mixture for studying the life history of these organisms should have the following properties: (*a*) The salt

solution should have a low oxygen content; (*b*) excess power of absorbing oxygen must be maintained to keep the amount of oxygen in the solution low during use; (*c*) it must have the correct pH—i.e., be nearly neutral; (*d*) the osmotic pressure of the solution must be correct; (*e*) the solution must not contain constituents toxic to the Protozoa.

Manganese hydroxide has the power of removing oxygen quite completely from solutions. In the reaction manganous hydroxide is oxidized to manganic hydroxide by the dissolved oxygen.

$$Mn(OH)_2(\text{solid}) + \tfrac{1}{4}O_2(\text{dissolved}) + \tfrac{1}{2}H_2O(\text{solvent}) = Mn(OH)_3(\text{solid}). \quad (1)$$

Both hydroxides are quite insoluble. This reaction is made the basis of standard methods of quantitative determinations of dissolved oxygen in water analysis (Winkler, 1888).

Although there is considerable confusion in the literature regarding the solubility of manganous hydroxide, the value taken is 0.00043 gram manganous hydroxide, or 4.8×10^{-6} mols per liter, determined by Almkvist (1918) for the solubility in distilled water at 22° C., without correction for dissolved carbon dioxide. These conditions are approximately those found under the conditions of use of a physiological salt solution. Considering manganous hydroxide as a strong base the pH of the saturated solution would be about 9. This value is indicated by the electrometric titration of manganous chloride with sodium hydroxide (Britton, 1925; Atkins, 1923), in which the precipitation of manganous hydroxide occurs between pH values of 8.6 and 9.2. It is evident by the application of the mass law to the reaction,

$$Mn(OH)_2(s) = Mn^{++} + 2OH^- \quad (2)$$

that an increase in the concentration of the manganous ion will decrease the concentration of the hydroxide ion by precipitating more solid manganous hydroxide. Computations will show that the pH of the solution is about 7.1 when the manganous concentration is 0.025 mols per liter and about 7.0 when it is 0.050 mols per liter.

Although work on the pH of the termite intestine (Randall and Doody, 1934) showed a pH of about 6.8 in the hindgut, the success of Cleveland with a solution of pH = 7.2 led to the use of a saturated solution of manganous hydroxide (with excess of the insoluble manganous hydroxide), having a concentration of manganous ion of 0.025 mols per liter (estimated pH = 7.1). The excess of the light flocculent manganous hydroxide furnishes a plentiful reservoir of oxygen remover to take care of any oxygen absorbed during the use of the solution

and acts as a buffer to retain the proper pH. There is no indication from the literature or from preliminary experiments with the manganous hydroxide that it should have any toxic effect on the intestinal Protozoa of the termite.

Mixing the Deoxygenating Solution

Boil more than one liter of distilled water vigorously for ten or fifteen minutes to remove most of the dissolved oxygen. It is best to use hot water in preparing the solution so that as little oxygen as possible will be redissolved. Dissolve 3.94 grams of manganous chloride ($MnCl_2$) in 25 ml. of the boiled water. To the remainder of one liter of the water (975 ml.) add 0.5 gram of sodium hydroxide. Pour this into a narrow necked liter flask which can be tightly stoppered. Add the manganous chloride solution, stopper tightly, and mix well by shaking. When cool, the deoxygenating solution is ready for use except that an adjustment for its osmotic pressure is required. The procedure as prescribed makes 0.56 gram per liter of insoluble manganous hydroxide, of which only 0.0000048 gram is dissolved. This provides a plentiful excess of the insoluble hydroxide. Always shake the mixture well before using, so there will be particles of the insoluble deoxygenating hydroxide in the solution which is to be used. As the dissolved manganous hydroxide is used it is replaced by more of the insoluble hydroxide going into solution.

The insoluble manganous hydroxide, $Mn(OH)_2$, is light pink in color. The manganic hydroxide, $Mn(OH)_3$, which is formed by reaction with the dissolved oxygen, is also insoluble; but its color is dark brown. This dark brown color appears during the preparation of the deoxygenating solution, due to the removal of the slight amount of oxygen dissolved in the water of the solution. This does no harm as there still remains an ample excess of the undissolved deoxygenating agent, manganous hydroxide. The solution should be kept in a tightly stoppered flask, as it deteriorates rapidly due to the absorption of oxygen from the air.

Adjustment for Osmotic Pressure

The osmotic pressure of the above saturated and buffered solution of manganous hydroxide is too low as was evident from the disruption of the cells of the Protozoa in preliminary experiments.

The salt mixture used by Cleveland was roughly equivalent to a 0.40 per cent solution of sodium chloride, which we found to give approximately the right osmotic pressure. Therefore 0.740 gram of sodium chloride should be added to each liter of the manganous hydroxide solu-

tion. A finer adjustment of the osmotic pressure is made from observations on the effect upon the Protozoa when the solution is used as a medium.

A more suitable medium for these Protozoa could probably be made by adjusting the osmotic pressure with one of the sugars which can be produced by the decomposition of cellulose. The hexoses ($C_6H_{12}O_6$) such as glucose, galactose, and mannose, can be produced from cellulose, and would probably show no ill effect in the intestine of an animal which lives on cellulose. The approximate adjustment of the osmotic pressure of the manganous hydroxide medium can be made by the addition of 6.67 grams of glucose, galactose, or mannose, or any mixture of them, to each liter of the medium.

Action on the Protozoa of Reticulitermes Hesperus

The method used by Dr. O. L. Williams of the Sub-committee on Biology, Termite Investigations Committee, to examine the Protozoa of *Reticulitermes hesperus* was as follows: The intestinal contents of a termite were mixed with about 0.5 ml. of the fluid medium containing manganous hydroxide (osmotic pressure adjustment made with sodium chloride). The depression in a hollow-ground slide was then filled with this dilution, and a cover-slip sealed in place with vaseline or paraffin.

Preparations made as above showed active Protozoa for periods of twenty-four hours or longer. Preparations made in the same manner with Cleveland's solution in place of the above described manganous-containing solution showed active Protozoa only during periods of less than two hours.

Mr. J. E. Gulberg, who has used the manganous solution in taking micro-motion pictures of the Protozoa *R. hesperus,* where the intense illumination required is a serious factor in limiting the survival of the Protozoa, found that it was possible to keep the organisms alive over a period of about two hours. This was accomplished without other special precautions to exclude oxygen from the preparations which were being photographed. Under the same severe conditions, with the usual physiological salt solutions, the organisms could not be kept alive more than a few minutes.

These results show considerable advantage for the preparations of the Protozoa from *Reticulitermes hesperus* in which the amount of dissolved oxygen is reduced. The manganous hydroxide mixture is itself a diluent of very low oxygen content. It also supplies a reserve oxygen-removing power which absorbs oxygen from the material which was diluted and maintains the mixture to be observed at very low oxygen con-

tent. The use of this manganous hydroxide mixture adds no complications to present technique and allows the production of essentially oxygen-free cultures more easily than with any of the methods in common use. The manganous hydroxide mixture as described above gives a buffered solution of adjustable pH and osmotic pressure which appears non-toxic to Protozoa.

Slight changes in the pH of the solution, the composition of the added salts, and the adjustment of the osmotic pressure can be made to suit other organisms requiring physiological salt solutions of low oxygen content.

We wish to make acknowledgment to the Termite Investigations Committee under whose auspices this work was done. We also take this opportunity to thank Mr. J. E. Gullberg and Dr. O. L. Williams for their coöperation.

BIBLIOGRAPHY

ALMKVIST, G., 1918. *Zeitschr. anorg. allgem. Chem.*, **103**: 240.
ANDREW, B. J., 1930. *Univ. Calif. Publ. Zool.*, **33**: 449.
ATKINS, W. R. C., 1923. *Trans. Faraday Soc.*, **18**: 310.
BRITTON, H. T. S., 1925. *Jour. Chem. Soc.*, **127**: 2110.
CLEVELAND, L. R., 1925*a*. *Biol. Bull.*, **48**: 282.
CLEVELAND, L. R., 1925*b*. *Biol. Bull.*, **48**: 295.
CLEVELAND, L. R., 1925*c*. *Biol. Bull.*, **48**: 309.
CLEVELAND, L. R., 1925*d*. *Biol. Bull.*, **48**: 455.
CLEVELAND, L. R., 1928. *Biol. Bull.*, **54**: 231.
RANDALL, M., AND T. C. DOODY, 1934. Chapter 8 of "Termites and Termite Control," University of California Press, Berkeley, California.
TRAGER, WILLIAM, 1934. *Biol. Bull.*, **66**: 182.
WINKLER, L. W., 1888. *Ber. deutsch. chem. Gesellschaft*, **21**: 2843.
WINKLER, L. W., 1889. *Ber. deutsch. chem. Gesellschaft*, **22**: 1764.

THE CHROMOSOMES OF HERMAPHRODITES

I. Lepas anatifera L.[1]

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Our information relative to chromosomes of hermaphrodite animals is still very scanty, which is deplorable in view of the fact that a general theory of the sex chromosome ought to be based on hermaphrodite as well as gonochorist forms. The only cases exhaustively studied are those of the nematode *Angiostomum* (*Rhabditis*) *nigrovenosum* (Boveri, 1911; Schleip, 1911) and the coccid *Icerya purchasi* (Hughes-Schrader, 1927, 1930). Both are atypical insofar as the "hermaphrodites" begin development as females, morphologically as well as with respect to chromosome numbers. When, later on, testicular islands and spermatozoa are formed, they arise only in consequence of a localized process of sex reversal which is accompanied by chromosomal regulations. In the case of *Angiostomum* one X chromosome becomes inactive and subsequently is omitted, while in *Icerya* the diploid set of chromosomes is reduced to the haploid. In either case, the chromosomal condition of the male is established. Indications of the regulation become visible at the first spermatocyte stage or even earlier. No somatic cells of male constitution are formed. Hermaphrodism, in these cases, is visibly secondary to the gonochorist condition.

There is good reason to assume that a vast majority of hermaphrodites are of a different type and have not been derived from gonochoristic ancestors. Thanks to data accumulated in papers by Boveri (1890), Stevens (1903, 1904, 1910), Buchner (1910), Elpatiewsky (1910) and Bordas (1912, 1914), we obtain a fairly complete picture of the chromosome cycle in several species of *Sagitta*. The diploid number of 18 chromosomes is found in somatic cells as well as in spermatogonia and ovogonia. Mature eggs and spermatozoa all contain 9. This situation corresponds to that which generally is considered to be characteristic for hermaphrodite and monoecious higher plants. In *Datura stramonium*, Belling and Blakeslee show that the diploid number is 24 and the haploid 12. That the chromosome sets of

[1] This investigation was supported by grants from the Committee for Research in Problems of Sex of the National Research Council.

pollen grains and eggs are qualitatively equivalent is demonstrated by genetical evidence. Hence there exists a well established type of hermaphrodism with sex differentiation that is not followed by chromosomal rearrangements in either sex. We designate this condition as primary hermaphrodism, since it appears to be the prototype from which the different forms of gonochorism in Metazoa and in higher plants have arisen.

In the hermaphrodite annelid *Ophryotrocha puerilis,* A. and K. E. Schreiner (1906) as well as Huth (1933) have demonstrated the presence of 8 chromosomes in somatic cells and in dividing gonia. In the maturation divisions of spermatocytes and ovocytes appear four chromatic units, and from Huth's careful morphological analysis one can conclude that all ripe gametes receive equivalent haploid chromosome sets.

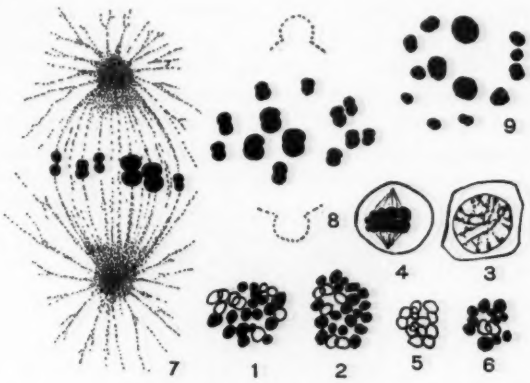
Perrot (1934), in his recent study on *Limnaea stagnalis,* gives 18 as the haploid number in both male and female maturing germ cells. The necessity of further critical studies in gastropod chromosome cycles is, however, emphasized by an earlier paper of the same author (1930).

In respect to the problem of the evolution of gonochorism the order of the Cirripedia would seem to be of particular interest. A large number of genera are straight hermaphrodites. Others consist of large hermaphrodites and smaller "complementary" males, while a few little studied species have large female and small male forms. The present paper deals with the goose barnacle, *Lepas anatifera* L., a common hermaphrodite. The material was collected at the Marine Biological Stations of Roscoff, France and of Woods Hole, Massachusetts.

In the intestinal walls of adult specimens relatively frequent mitoses are found which lend themselves favorably to chromosome counts due to the large size of the cells. Prophase and metaphase chromosomes appear like beads of different size, the largest measuring about eight times the volume of the smallest ones. In the equatorial plate these chromosomes are crowded together, though in favorable cases one can easily enough establish 26 as the diploid somatic chromosome number (Figs. 1 and 2).

Spermatogenesis is characterized by the extreme smallness of the cells. The successive stages of maturation of primary spermatocytes were studied with utmost care. Slides were stained with hematoxylin after Ehrlich, Delafield and Heidenhain as well as by the Feulgen method. The chromosomes unravel and form very fine threads during the conjugation phase (Fig. 3). Heteropycnotic elements distinctly are absent. Due to the small size of the spindle, the chromosomes of the first meiotic metaphase are so closely packed that they can be in-

dividually seen and counted in a small fraction only of the innumerable dividing spermatocytes that one finds in testes during the reproductive season (Figs. 4–6). If seen in profile (Fig. 4), the chromosomes appear as paired beads. As they segregate and move toward the spindle poles they soon become rearranged, some moving faster, others slower. The minute size of the spindle simply does not permit the



FIGS. 1–9. *Chromosomes of Lepas anatifera L.* 1, 2, dividing intestinal cells. 3, spermatocyte in conjugation phase. 4–6, spermatocytes in first maturation division. 7, profile view of first meiotic spindle in the egg. 8, chromosomes in a first meiotic spindle which stands obliquely in the section. 9, polar view of the chromosome plate of the first meiotic spindle in the egg. All figures × 2000.

advance of undisturbed chromosomal plates during anaphase. No uneven distribution of the chromosomes is indicated and no chromatin elements are eliminated. The author was not able to count chromosomes in the second meiotic division, though it was ascertained that no chromosome elimination occurred here, either.

Lepas, like many other crustaceans, brings periodically large numbers of eggs to simultaneous maturity. In one specimen the ovary contained many thousands of eggs, all at the stage of metaphase of the first maturation division. The spindle which lies close to the surface of the oval egg has more than three times the linear dimensions of the corresponding spermatocyte figure (compare Figs. 7 and 4). The chromosomes are well separated since they are relatively smaller (barely twice the diameter of spermatocyte chromosomes; Figs. 9 and 6). The haploid number of 13 was established with greatest ease in more than two hundred individual counts. Three large chromosomes generally occupy the center of the plate while ten smaller ones usually take their

place along its periphery. The relative size differences are the same in ovocytes and spermatocytes. The fact that the individual chromosomes are at least four times as voluminous in female as in male germ cells of identical stage suggests that the visible chromatin represents much more than the aggregate volume of the genes.[2] Seen in profile the metaphasic chromosomes appear as pairs composed of two equal elements (Figs. 7 and 8).

Weismann and Ischikawa (1888) had seen " four double-chromatin-elements " in the maturation spindle of the egg, while Groom (1894) reports that " the number of chromatic elements varies; it appears to be commonly four or five, but, in some cases was, as far as I could make out, as many as ten or twelve." To prove the incorrectness of these counts we refer to the photomicrograph Fig. 10. The true number

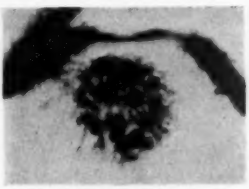


FIG. 10. Photomicrograph of the 13 chromosomes in the first maturation spindle in the egg of *Lepas*. × 1500.

can be ascertained without difficulty even in properly stained whole mounts.

No heteromorphic sex chromosomes have been found in somatic or in germinal cells of either sex. Nor have we observed any indication of chromosomal regulation of either the *Angiostomum*- or the *Icerya*-type. Even though this study does not include some of the important phases of the chromosome cycle (especially that of fertilization), the evidence obtained renders it more than probable that the goose barnacle is a primary hermaphrodite in the sense defined above. Delage (1884), G. Smith (1906), Meisenheimer (1921), and others have defended the opinion that hermaphrodism in *Cirripedia* is secondary, the order having descended from gonochoristic, free-swimming ancestors. The evidence of comparative morphology has, however, been interpreted also in the opposite sense. The chromosomal situation is distinctly in favor of Darwin's (1851, 1854) theory of primitive hermaphrodism and second-

[2] In *Ophryotrocha*, according to Huth (1933), the chromosomes of the polar spindle are of same size as those of the first spermatocyte, even though the spindles show a similar size ratio as in *Lepas*.

ary development of "complementary" males and of complete gonochorism.

BIBLIOGRAPHY

BORDAS, M., 1912. Contribution à l'étude de la Spermatogénèse dans la Sagitta bipunctata. *La Cellule,* **28:** 165.

BORDAS, M., 1914. Doctrinas actuales sobre la reduccion numérica de los chromosomas y su aplicacion á la espermatogénesis de la Sagitta bipunctata Quoy et Gaim. *Mem. d. l. Real. Soc. Expan.,* **10:** 1.

BOVERI, TH., 1890. Zellen-Studien. Über das Verhalten der chromatischen Kernsubstanz bei der Bildung der Richtungskörper und bei der Befruchtung. *Jena. Zeitschr. f. Naturwiss.,* **24:** 314.

BOVERI, TH., 1911. Über das Verhalten der Geschlechtschromosomen bei Hermaphroditismus. Beobachtungen an Rhabditis nigrovenosa. *Wuerzburg Verh. physik. Ges. (N.F.),* **41:** 83.

BUCHNER, P., 1910. Die Schicksale des Keimplasmas der Sagitten in Reifung, Befruchtung, Keimbahn, Ovogenese und Spermatogenese. *Festschr. f. R. Hertwig., Jena,* **1:** 233.

DARWIN, C., 1851–54. A Monograph of the Sub-class Cirripedia. The Lepadidae, London 1851; The Balanidae etc., London 1854.

DELAGE, Y., 1884. Évolution de la sacculine. *Arch. Zool. expér. et gén., 2 sér.,* **2:** 417.

ELPATIEWSKY, W., 1910. Die Entwicklungsgeschichte der Genitalprodukte bei Sagitta. I. Die Entwicklung der Eier. *Biol. Zs. Moskva,* **1:** 333.

GROOM, T. T., 1894. V. On the Early Development of Cirripedia. *Phil. Trans. Roy. Soc. London, Ser. B,* **185:** 119.

HUGHES-SCHRADER, S., 1927. Origin and Differentiation of the Male and Female Germ Cells in the Hermaphrodite of Icerya purchasi (Coccidae). *Zeitschr. f. Zellforsch. u. mikr. Anat.,* **6:** 509.

HUGHES-SCHRADER, S., 1930. The Cytology of Several Species of Iceryine Coccids, with Special Reference to Parthenogenesis and Haploidy. *Jour. Morph.,* **50:** 475.

HUTH, W., 1933. Zur Cytologie der Ophryotrochen. *Zeitschr. f. Zellforsch. u. mikr. Anat.,* **20:** 309.

MEISENHEIMER, J., 1921. Geschlecht und Geschlechter im Tierreiche. Bd. I. Jena.

PERROT, J. L., 1930. Chromosomes et Hétérochromosomes chez les Gastéropodes pulmonés. *Rev. Suisse Zool.,* **37:** 397.

PERROT, J. L., 1934. A propos du nombre des chromosomes dans les deux lignées germinales du Gastéropode hermaphrodite Limnaea stagnalis (var. rhodani). *Rev. Suisse Zool.,* **41:** 693.

SCHLEIP, W., 1911. Das Verhalten des Chromatins bei Angiostomum (Rhabdonema) nigrovenosum. *Arch. Zellforsch.,* **7:** 87.

SCHREINER, A., AND K.E., 1906. Die Reifung der Geschlechtszellen von Ophryotrocha puerilis Clprd.-Mecz. *Anat. Anz.,* **29:** 465.

SMITH, G., 1906. Rhizocephala. Fauna Neapel, Berlin, **29:** 1.

STEVENS, N. M., 1903. On the Ovogenesis and Spermatogenesis of Sagitta bipunctata. *Zool. Jahrb., Abt. f. Anat.,* **48:** 227.

STEVENS, N. M., 1904. Further Studies on the Ovogenesis of Sagitta. *Zool. Jahrb., Abt. f. Anat.,* **21:** 243.

STEVENS, N. M., 1910. Further Studies on Reproduction in Sagitta. *Jour. Morph.,* **21:** 279.

WEISMANN AND ISCHIKAWA, 1888. Weitere Untersuchungen zum Zahlengesetz der Richtungskoerper. *Zool. Jahrb. Abt. f. Anat.,* **3:** 575.

CENTRIFUGING THE EGGS OF ILYANASSA IN REVERSE

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Introduction

A study of the factors involved in the formation of the so-called yolk-lobe or antipolar-lobe in the snail *Ilyanassa* carried out in the summer of 1932 led to certain provisional conclusions concerning the nature of the lobe. Most of the experimental work was carried out by centrifuging the eggs at different stages previous to and during the formation of the lobe. The main problem was to discover whether the lobe is only an expression of changes taking place elsewhere in the egg —changes that are directly or indirectly correlated with mitosis—or whether the lobe is, in some sense, a predetermined region of the egg, and its formation merely incidental to the other phases in the mitotic or other phenomena. The view presented in the following pages is that neither of these interpretations is strictly true, because while the lobe region may be regarded as present in the ripe eggs, its delineation is not strictly preformed, and to a limited extent it possesses a certain autonomy of its own, and is independent of the mitotic phenomena as such. It is even independent of the materials contained inside that part of the egg. It should be recalled in this connection that the first time the lobe appears (Plate I, 1) it is scarcely recognizable, and can not be said to be constricted from the rest of the egg. The egg merely becomes pear-shaped with a pointed or rounded region barely outlined at the antipole. On the other hand, when the second lobe appears the lobe is a conspicuous part of the egg (Plate I, 2–7), but it never becomes much constricted at the base from the rest of the egg. The third lobe (Plate I, 9–13) at first resembles the second very closely, but later it is completely constricted from the egg when the first division is completed. The fourth lobe (Plate I, 17–18) is little more than the constriction near the middle of one (the larger) of the dividing cells. Subsequently one of the two cells is pinched off from the yolk, the other remaining attached to the yolk by the constricted region (Plate I, 18–19). This cell soon fuses with the yolk (Plate I, 20), giving the typical four-cell stage.

Between lobe-forming stages, the lobe may be said to return into

the egg. This is true at least after the first and second lobe formation, while the third lobe fuses with one of the first two blastomeres (Plate I, 15–16) which does not become spherical. The fourth lobe remains as a part of one of the four blastomeres, fusing with it into one large oval cell (Plate I, 17–20).

It may also be recalled that when the first lobe appears the first

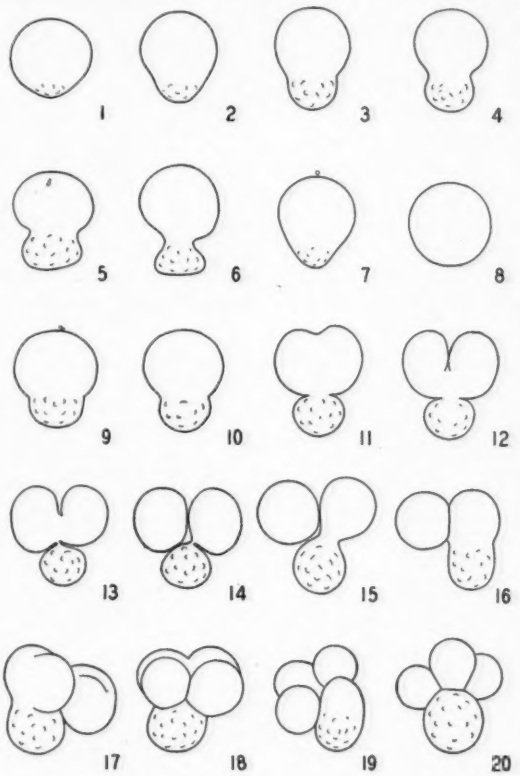

PLATE I

polar body is coming off. The mitotic figure at the pole is very small at this time, although before passing to the pole it was quite extensive. On the other hand, the second lobe is large. At the time of its beginning the second polar body is being extruded. The mitotic figure of the second polar body is no larger than that of the first. This lobe continues to grow larger, during which time the egg-pronucleus is de-

veloping, and the sperm-pronucleus is approaching to fuse with it. When the third lobe develops, there is a very large mitotic figure in the upper hemisphere. The lobe is fully delimited at the time when the cleavage begins to cut into the upper hemisphere.

It is obvious that the extent, and in some degree the size of the lobe, is not intimately bound up with a particular stage of the mitotic figure, and that it is a more or less independent development in the antipolar hemisphere of the egg.

By means of centrifuging the eggs upside down ("in reverse"), I attempted to find out whether the formation of the lobe is due to materials contained within it, and whether its location is dependent on the location of the mitotic spindle. It has been surmised for other forms—leeches, molluscs and polychaetous annelids whose eggs also develop lobes somewhat like those of *Ilyanassa*—that the lobe contains preformed material essential for certain later embryonic structures. If this is correct, then it might seem to follow that the formation of the lobe itself is connected with the presence of these materials in the antipolar hemispheres. The experiments that I have carried out show that this is not the case, since the lobe will still develop when its materials have been removed and carried into the polar hemisphere. It may, of course, be true that "organ-forming substances" may be present in some of these eggs with lobes as has been claimed, but even if this is true it does not seem probable in the light of the experiments that the mechanics of lobe-formation has anything to do with the presence of such materials. This statement does not mean that there are not preformed structures in the antipolar hemisphere that are involved in lobe-formation. I am inclined to believe that in the surface layers of this region, that can not be removed by centrifuging, the key to the situation may be found. In support of this view is the fact that when the upper hemisphere is pinched off from the rest of the egg by centrifuging in raffinose, the cleavage of this half is into two and four equal cells. No lobe appears. The other half, the lower hemisphere, or the "bottom" will develop a lobe, even when the female and male nucleus are absent.

There are three separate but interdependent topics discussed in this and two following papers: the behavior of the egg when its interior materials are partly or wholly reversed; the cleavage of the "tops" and of the "bottoms" after their separation; the rhythmic formation of the lobe, either in the presence of the egg-chromatin alone (the sperm-nucleus having been removed), or in the total absence of all chromatin.

Eggs Centrifuged in Reverse

Introduction

The chromatin in eggs that have just been laid and centrifuged before the first polar body has been extruded is, in the inverted egg, carried along one side, or even into the antipolar hemisphere. The sperm-pronucleus is also carried along with the protoplasm and can sometimes be found in the protoplasmic zone. Occasionally I have seen a polar body given off from the clear zone, but since the polar bodies have not often been seen in these eggs, when alive, it may be that the chromosomes of the polar spindle often divide, but do not separate into two nuclei and remain in the egg. There are some indications that a large number of the chromosomes are present when the segmentation spindle forms later at the side or in the antipolar hemisphere, but the chromosomes are so small and clumped that I have not been able to count them.

An important consideration is whether any protoplasmic readjustments take place in the inverted or in the partly inverted eggs after removal from the centrifuge. In general the whole of the protoplasm with its contained chromosomes has been carried along one side of the egg, and in completely inverted eggs it is in this way carried to the antipole. The oil before centrifuging is contained largely in the protoplasmic substance of the polar hemisphere, giving it an opaque or brownish appearance by transmitted light, or white in direct light. In well centrifuged eggs the oil leaves the protoplasm and collects as a segment at the centripetal pole. The protoplasm that lies beneath is left as a clear zone or band which stains a deep blue in haematoxylin preparations. The impression one gets when the centrifuged eggs are examined is that in some of the inverted eggs the protoplasm and oil have been carried completely to the centripetal end of the egg, while in others it has not yet been carried so far, but lies at the side. This impression is erroneous, as shown by continuing the centrifuging. It is then found that the conditions have not changed, but the same proportion of eggs still have the protoplasm at one side. In fact, the shift takes place in the first two or three minutes. The explanation is that only some of the eggs are completely inverted at the start, while many of them lie obliquely. An examination of the "inverted" eggs (in which the lobe is present) in the gelatin after it has been congealed confirms this conclusion. For example: eggs that had been "inverted" in the gelatin which was then solidified by cooling were observed in a dish of ice-water under the microscope. These eggs had a large second polar lobe, which made it possible to determine their orientation in the tube. Before centrifuging, 28 eggs were completely reversed, 59 were

oblique, and 2 were not reversed at all. After centrifuging 31 were recorded as completely reversed, 2 not at all reversed, and the rest were oblique to the centrifugal force (some of these lay on their sides, Plate II, 2). This means that, as the eggs sink in the gelatin before it is frozen, some of them are completely inverted, while others lie obliquely and are centrifuged in this position. In the latter, the protoplasm slides along one side of the egg and spreads out there. The situation can be more accurately described by saying that the whole mass of yolk moves along one side, viz., towards the centrifugal pole, forcing the lighter protoplasm along the opposite side, i.e., toward the centripetal pole. When it is remembered that, unless an egg is perfectly oriented with its pole outwards, this sort of shifting is expected to take place, there can be no question but this represents the actual situation when the centrifugal force is applied. Whether in a perfectly reversed egg the yolk will be driven through the center of the egg and the protoplasm around the sides (as a ring), or whether the two materials literally pass through each other on the way to their respective poles, can not be positively asserted, but if the latter happens I have seen no evidence of it. Certainly such a method of rearrangement in the egg of *Ilyanassa* must be very exceptional.

In other kinds of eggs, where the protoplasm and yolk are more generally mixed, it is believed that the yolk granules pass individually through the protoplasm to reach the centrifugal pole and the oil droplets pass in the opposite direction. This may be, of course, what happens in such eggs, but there are indications in some of them at least, especially when the segmentation spindle is present, that the protoplasmic portion tends to remain more or less intact, and is shifted as a whole, while the yolk passes along one side toward the centrifugal pole. However this may be for other eggs, in the molluscan egg where the two regions are rather sharply defined, the rearrangement takes place by the shifting of polar and antipolar materials as wholes around and not through each other. In the frog's egg also, where the upper and lower hemispheres are well delimited, there is evidence that when the egg is not completely inverted the regions shift around each other as wholes in response to gravity.

This consideration has some interest in connection with the possible shifting back of the materials that are incompletely inverted so that the protoplasm returns to the polar end of the egg, as Conklin has described for *Crepidula*, especially in cases where the polar bodies are given off at the pole and after the protoplasm and the polar spindle have been carried away from this region. It is to be expected that different eggs may behave differently in this respect, but in *Ilyanassa*

at least there is no evidence that a return movement takes place except to a minor degree. The protoplasm may concentrate around its new center after the eggs are removed from the machine, and may be supposed, if the centrifuging is not complete, to concentrate at times at one side of or even in the polar region; but the evidence from *Ilyanassa* gives little support for the view that there are any striking readjustments of this kind. When eggs with much yolk concentrated at the antipole are centrifuged in mass in the bottom of the tube, most of them will lie, at first, obliquely to the centrifugal force, and when they are not free (due to mutual pressure) to orient with the heavier end outward, the rearrangement of the contents must be like that in *Ilyanassa,* i.e., along the side. As I have said, if the centrifuging is only partial it may happen sometimes, when the greater mass of the cytoplasm remains at or near the pole, that it concentrates again near the pole. But this is very different from the implication that there is some attracting pole " force " that brings this about. The viscosity of the interior protoplasm and that of the inner layer of the surface of the egg may account for whatever rearrangement takes place.

A few statements additional to those given in my previous paper may be made with respect to the congealed gelatin technique. By using an inner tube a little larger and longer than the ones used before, a longer time is allowed for the sinking eggs to become inverted, and a higher percentage of the eggs are reversed. The outer large glass centrifuge tube should be completely filled with cracked ice, and the inner tube imbedded in this ice. Some of the ice will remain unmelted for about five minutes, especially if the brass tube of the centrifuge and the outer glass tube are cooled beforehand. When a longer time is needed, the centrifuge can be stopped, the small tube containing the eggs taken out, and a new supply of ice added. Since the eggs are in a solid medium they will not change their orientation in the interval. It is important that the smaller tube be completely filled with gelatin after the eggs are in it, so that when the forefinger is placed over it, and the tube turned over, no air bubbles are left; for if present they rise through the gelatin (then liquid) to the top, when the tube is turned over, and when the centrifuging begins they may be forced back to the centripetal end and disarrange the eggs. A little sea water is left with the eggs at the bottom of the tube when the liquid gelatin is added above it to fill the tube to the top. The eggs will not sink quickly out of this water into the gelatin unless the inverted tube is violently shaken downwards. This starts the eggs in the gelatin, and also breaks up clumps of eggs. Once started they will slowly sink in the gelatin if it is prevented from freezing by holding the other hand

around the tube at this time. After a couple of minutes the eggs will be distributed along the whole length of the tube. Should the gelatin melt in the tube while on the centrifuge, the eggs may become oval, but this does not happen as long as the gelatin remains congealed. Also, in removing the centrifuged eggs from the small tube it is important not to deform them by drawing out the gelatin too rapidly before it is liquid again. This is avoided, after warming the tube in the hands, by holding the tube obliquely with the open end under warmed sea water in a flat dish, and by sending small bubbles of air upward from a pipette into the gelatin to replace it. The eggs slowly pass with the gelatin into the sea water. They must then be gently agitated to remove the gelatin and to mix it with the sea water, which is then withdrawn and replaced with new sea water.

The nuclei and chromatin of the early stages are difficult to differentiate. After Bouin's solution the haematoxylin preparations are not so good as after picrosulphuric acid, since in the former the yolk holds the stain. The most successful preparations were stained in Conklin's formula for Delafield's haematoxylin, viz., by diluting it six times with water and adding one drop of picrosulphuric acid to each 10 cc. of diluted stain. After ten minutes in the stain the eggs are washed for half a minute or less in acid-alcohol diluted to one-fourth with 70 per cent alcohol, then run up through the alcohols, etc. to balsam.

The Experiments

Repeating the experiments of the previous year, many sets of eggs were centrifuged in reverse. Preparations were made of some of these to detect the location of the egg-chromatin, the sperm-pronucleus, and the polar bodies. In eggs newly laid, the chromatin was always found in the protoplasmic zone. Since at this time there are no orienting points, it is not possible to state how many of the eggs were completely reversed. In some cases the polar bodies were seen to come off later at the side of the clear area, but it is uncertain whether or not these are eggs in which the protoplasm has been only a little displaced, and the evidence is insufficient to show that the polar bodies may be extruded at the side when the protoplasmic zone has been carried as far as and into the antipolar hemisphere. Since polar bodies were seen in relatively few cases, it follows that they are not always given off whether the reversal is complete or only partial. The yolk-lobe appeared both in those partially and in those completely reversed—the latter containing all or only part of the oil at the antipode. Some of the records of these eggs are as follows:

Newly laid eggs were centrifuged at 2,500 r.p.m. for 10 minutes in reverse (new ice added after 5 minutes). In the control the first polar

body came off 10 minutes later. The second polar body was seen coming off the side of the clear zone in some of the centrifuged eggs (Plate II, 1). When the second lobe appeared it was evident that the protoplasm in many eggs lay at one side of the egg (Plate II, 2).

Only a few sets of eggs were centrifuged in reverse before the first polar spindle had come to the surface, since these stages were sufficiently examined in the two previous summers' work. In the absence of the polar bodies or polar spindles at the surface there are no points by which to orient the true antipolar hemisphere. In some cases, as reported in 1933, polar bodies came off from some of the eggs, that were only partially reversed, in the polar hemisphere, but whether exactly at the pole or near the pole in the polar hemisphere could not be definitely stated. In such cases the second lobe evidently came off at the antipole. In eggs completely reversed the chromosomes are carried into the antipolar hemisphere, as determined by the location of the second lobe. There were no cases found in which polar bodies were present in that hemisphere. The lobe developed as in the other cases. The new experiments now to be described begin with stages when the first polar spindle was at the surface, and the polar body about to be extruded.

These experiments with eggs, in reverse, at the time when the first polar body or the second polar body was just coming off, were made in order to see whether the polar spindle remains sticking at the pole when the protoplasm is driven to the side or to the antipole, and also to see, if this happens, whether the second lobe forms at the antipole. The results are not, on the whole, satisfactory, because in the first place the spindle was carried away in some cases with the protoplasm; in other cases it remained at the pole; and because, in the second place, the first polar body when free is sometimes lost and then does not serve as a marker, or becomes very difficult to detect in eggs that are reversed. In the eggs that were not reversed, hence have the pole inwards, the polar body remains attached as a rule, and stands out conspicuously from the surface of the egg. On the other hand, when the egg is reversed, the polar bodies appear often to be lost, or become difficult to detect if they lie at the outer end of the reversed egg, or at the sides of eggs partly reversed.

A set of newly laid eggs was centrifuged at 2,500 r.p.m. in reverse for 5 minutes, at the time when the first polar body was just out. The second lobe appeared 19 minutes later, both in the control and in the centrifuged eggs. The second polar spindle was then at the surface just below the polar body. Centrifuged eggs killed at once showed that the first polar body had remained at the pole. In eggs not reversed, Plate II, 3, it was above the polar protoplasm; in eggs completely reversed it lay, when found, opposite the oil (Plate II, 4); in others

partly reversed (Plate II, 6) it lay at the side. In all these except that shown in Plate II, 5, the second metaphase plate was found near the first polar body.

Three other sets were centrifuged when the first polar body was coming off. The spindle was displaced in some of the eggs and carried with the protoplasm and oil even to the antipole. The results were the same as in the last case.

Eggs were centrifuged for 5 minutes when the second lobe had just appeared. They were killed as soon as removed from the machine. One partly reversed egg is shown in Plate II, 7. This egg had lain on its side. The first polar body is at the pole (possibly this is the second polar body): the protoplasm is on one side extending into the lobe; the egg-chromosomes are in the protoplasm above the equator, and the sperm-nucleus at the equator.

Another set of eggs was also centrifuged for 5 minutes, just as the second lobe was beginning. Some were killed and stained 13 minutes later (Plate II, 8–10). The first polar body was present at different points of the surface with respect to the stratification; the second spindle in metaphase lay just below it. Here the second spindle had not been shifted when the protoplasm was carried to the side or even to the antipole. In both cases the lobe developed later at the antipole. The protoplasm had not moved back to the region where the second spindle lay when the eggs were removed from the machine.

Eggs were centrifuged for 5 minutes in reverse after the first polar body had been extruded, just as the second lobe was appearing. Nineteen minutes later the lobe was fully out. The oil and protoplasm were either in it, or at the side in most eggs. When the lobe contained the oil, it was later constricted off as a rule. When protoplasm and oil were at the side, and the eggs began to round up, the lobe was not constricted off and returned to the egg. The third lobe appeared in these eggs, even when it contained most or all of the oil field. Later still, 14 eggs divided into 2 cells, mostly those in which the oil was at the side. Twenty-one eggs did not divide, 11 of which remained spherical, and 10 developed lobes (6 containing yolk and 4 oil). It is probable that the spindle had remained at the pole in these eggs, but I am unable to prove this. If so, it may not have been in position to induce cleavage because protoplasm was lacking, or because the sperm-nucleus may have been so far removed from the egg-chromatin that no cleavage spindle was formed.

Eggs were centrifuged for 5 minutes in reverse when the second lobe was coming out. It was still present when the eggs were removed from the centrifuge. In those which were actually reversed the oil con-

stricted off later and remained outside, while in those not reversed or partially reversed it remained as part of the egg. When the third lobe appeared 13 eggs divided at one side (Plate II, 11); 7 with oil in the

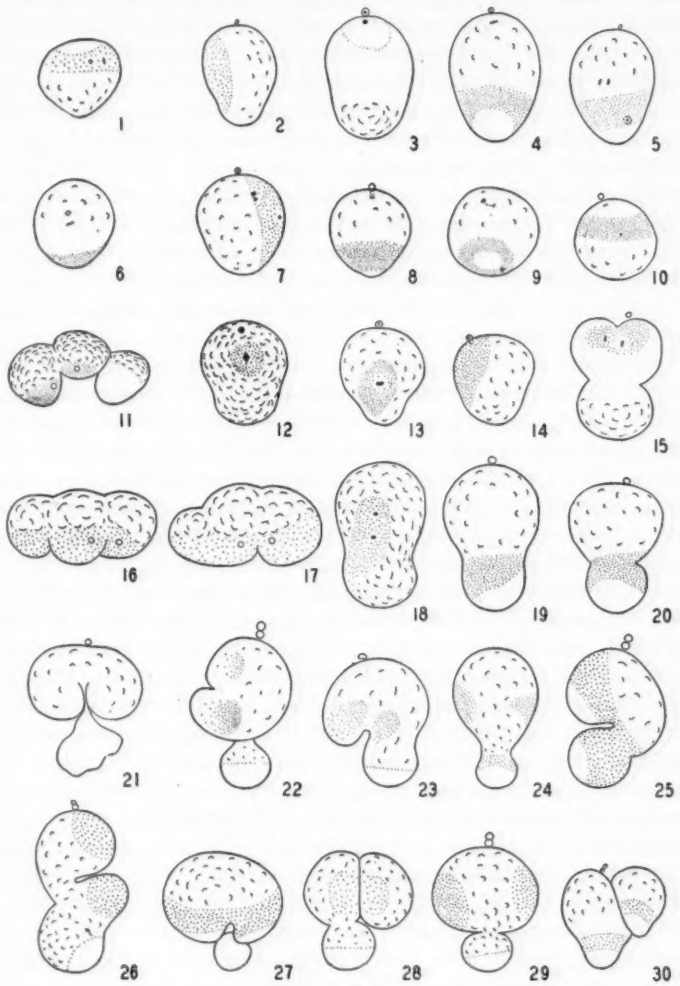

PLATE II

lobe did not divide; 4 with yolk in the lobe did not divide, and 19 remained spherical.

Eggs were centrifuged in reverse when the second lobe was halfway

out. It was still present in almost all of the eggs when taken from the centrifuge. The protoplasm was in almost all of the eggs at the side; in one it was in the lobe. The lobe went back in all eggs, and reappeared later. Three eggs are represented at this time in Plate II, 12–14. In these the polar body is present at or near the true pole—the lobe is at the opposite side, the protoplasm and oil are at the side, the chromosomes are present in the middle of the protoplasm.

Eggs were centrifuged as the second lobe was just coming out. The lobe was well out 13 minutes later, and was still well developed 10 minutes later. Except in those eggs with all the oil in the lobe, the lobe went back, and about an hour later the third lobe appeared. This set was killed and drawn when the cleavage was coming on (Plate II, 15). In 15, in an egg not reversed, the division began at the pole; in Plate II, 16, 17, at one side; in the others the nucleus had divided (telophase, Plate II, 18) but the protoplasm had not constricted. The lobe is conspicuous in all of these.

Eggs with large third lobes were centrifuged for 5 minutes. The controls had begun to divide 10 minutes later. At this time in four of the centrifuged eggs (Plate II, 19, 20) the oil is in the end of the lobe, with a clear protoplasmic band beneath. The yolk is in the polar hemisphere. The controls were in two cells, 10 minutes later, but the reversed eggs did not show indications of division until two hours after removal from the centrifuge. One hour later the lobe, when it contained the oil, constricted off from the rest of the egg, and the eggs began to cleave at or near the point at which the oil (now constricted off) is present, as seen in Plate II, 21. It is into this region that the mitotic figure has been driven. In most of the eggs the constriction cut through 20 minutes later, dividing the egg into equal or nearly equal parts. Later the eggs divided irregularly. It will be noted that neither the protoplasm nor the yolk moved back to the pole in these eggs—in fact, the cleavage was about due when the eggs were centrifuged.

A few experiments were carried out in reverse when the eggs were starting to divide in order to drive the mitotic figure into the antipolar hemisphere. The point of interest here is to see if the cleavage begins at the pole, or at the new location of the spindle. Four reversed eggs are shown in Plate II, 22–25. In 25, part of the oil is in the lobe and part in the antipolar end of what would have been one of the two blastomeres. In both parts there is a clear zone next to this, containing no doubt the chromosomes. The yolk is at the pole near the two polar bodies. In 23 the conditions are similar. In 22 the oil is in the lobe, but the clear area is at the side of the bilobed region. In 24 the oil is in the lobe, and below it there is some of the clear protoplasm. There

are two clear zones on the sides of this egg. Evidently most of the protoplasm of the dividing egg, already separated into two regions, has been driven along the opposite sides of the egg. The individual differences in these cases are due in part to the stage of the egg at the time of centrifuging, and in part to the angle which the partially reversed egg made with the centrifugal force. It is evident, nevertheless, that the lobe still retains its shape, more or less, when the yolk is driven out, and some of the oil and protoplasm into it, even after the egg is removed from the semi-solid jelly. Ten minutes later the condition of four eggs is shown in Plate II, 26–29. In 28 the egg has begun to divide at the side; the lobe contains the oil; in 26 the division is incomplete; in 27 the cleavage furrow begins near the antipole where the remains of the lobe containing most of the oil is present. No new lobe is formed opposite the cleavage furrow—i.e., at the true pole. In 29 the division was suppressed, the protoplasm is on opposite sides, the oil is in the lobe.

Eggs in the late two-cell stage were centrifuged for six minutes at 1,840 r.p.m. in reverse. The two cells were stratified independently of each other without altering to any great extent the shape of the cells (as shown in Plate II, 30), except in so far as the polar end of the larger cell, out of which the yolk was driven, became broader and the antipolar end more pointed—the reverse of the previous condition.

These results, with eggs about to divide, show the stability of the form of the egg during centrifuging in the jelly. This is perhaps to be expected, since the egg is held in a solid medium. On the other hand, when the gelatin is melted and the eggs are returned to sea water they retain the same form despite the new distribution of the interior materials. It appears that the form is determined by the surface layer that is affected very little or not at all by the centrifuging. The results show that the normal cleavage furrow fails to develop when the protoplasm and the mitotic figure are driven from the pole. Later a furrow may appear above the secondary position of the mitotic spindle. But in the last category there were no cases where the furrow cut through the center of the lobe of the egg, which remained at one side or was eliminated with the oil field. This indicates that the surface of the antipolar region of the egg is different from the surface of the rest of the egg, a conclusion in harmony with other results described in my previous paper.

THE SEPARATION OF THE EGG OF ILYANASSA INTO TWO PARTS BY CENTRIFUGING

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Before giving the detailed experiments, in which the top of the egg (the polar region) is separated from the bottom (the antipolar region) by means of centrifuging in sugar solutions of various compositions, a few general points may be mentioned.

The relative size of the tops that come off depends in part on the condition or stage of the egg at the time of centrifuging, but largely on the medium in which the egg is centrifuged. In an isosmotic cane sugar solution, or even in stronger solutions, the tops are usually small. In a nearly saturated solution of raffinose the tops are generally quite large, and contain nearly all the protoplasm of the egg.

The spermatozoön enters the normal egg at any point on the surface. It is not easy to demonstrate the sperm-head in the newly laid egg, for as long as it lies at the surface of the egg it is quite small. At the time of extrusion of the polar bodies it is easier to find, since the sperm-head is larger, but it is still at the surface. The sperm-head enters the upper hemisphere more frequently than the lower. It does not sink deep into the protoplasm until after the second polar body is out. In newly laid eggs the germinal vesicle is sometimes present, but more often it is just breaking down when its large nucleolus is set free in the protoplasm and may, without some experience, be easily mistaken for the sperm-pronucleus at this time. The nucleolus disappears, however, before the sperm-nucleus approaches the egg-pronucleus.

In the centrifuged egg the sperm-nucleus is carried to the inner pole and lies in the protoplasm there, or on the border of the oil field. It is generally driven off with the protoplasm and oil when the top is large. I have seen very few cases where it is left with the bottom when a large part of the upper half of the egg has constricted off. Since the sperm-pronucleus may lie at first in any part of the egg, it is rather surprising that it appears always (?) to be carried to the centripetal end. In some other eggs, *Urechis,* for instance, the condensed sperm-head is heavier than the rest of the egg and is found most often near the pigment pole; but in *Ilyanassa* it is lighter and remains with the protoplasm. When a large top is thrown off it sometimes contains both the

egg-chromatin and the sperm-pronucleus, since it gives off polar bodies and in many cases divides later. Such tops may, however, contain only the egg-chromatin. These give off polar bodies but do not divide. Here the sperm-nucleus appears to be absent, either because it is driven out of the top (with some of the oil), or because it is left behind in the bottom. Larger tops divide more often than smaller ones. Conversely, when only a small top is driven off, it may contain the sperm-nucleus, leaving the egg-pronucleus in the bottom. Under these conditions the bottom is not expected to divide, since it lacks the sperm-center. Such bottoms I have found in stained preparations. They produce lobes that are like the normal second lobe in every respect, and the lobe is better developed than the lobes of small bottoms that lack all chromatin. Many bottoms do not contain any of the egg-chromatin. Nevertheless, as will be described, they too form lobes. I have not succeeded in identifying any bottoms that had lost the egg-chromatin but retained the sperm-nucleus. Some such bottoms are perhaps to be expected, especially when the sperm happens to enter the antipolar hemisphere. I had hoped to identify monospermic fragments by the reduced number of chromosomes, but owing to the smallness of the chromosomes I have not succeeded in counting them. The possible occurrence of such bottoms does not affect the main issues raised here.

Centrifuging in Cane Sugar Solution

Eggs taken from a capsule that is just being laid were centrifuged at 2,680 r.p.m. for 9 minutes in a cane sugar solution consisting of 450 gm. sugar in one liter of tap water. The tops consisted of an oil cap, a clear zone, and a little fine yolk beneath (Plate I, 2). The tops were much smaller than the bottoms (Plate I, 1). In the control the second lobe began 17 minutes later, and in the tops the first polar body was coming off when removed, or a little later. It comes off usually at one side of the oil (Plate I, 4) or near its center. No lobes appear in the tops at any time. The controls divided 3 hours after the eggs were laid. Half of the tops divided at about this time into equal cells. They went into a four-cell stage one hour and 12 minutes later (Plate I, 6) and after one hour and 38 minutes into 8 cells with micromeres (Plate I, 7). The bottoms had gone to pieces (absorbed water) in this set.

In another set, just laid, the eggs were centrifuged in the same solution for 7 minutes at 2,680 r.p.m. The tops were larger than in the last set (compare Plate I, 14 and Plate I, 4). The bottoms were drawn out (Plate I, 8–9) with a neck containing clear protoplasm.

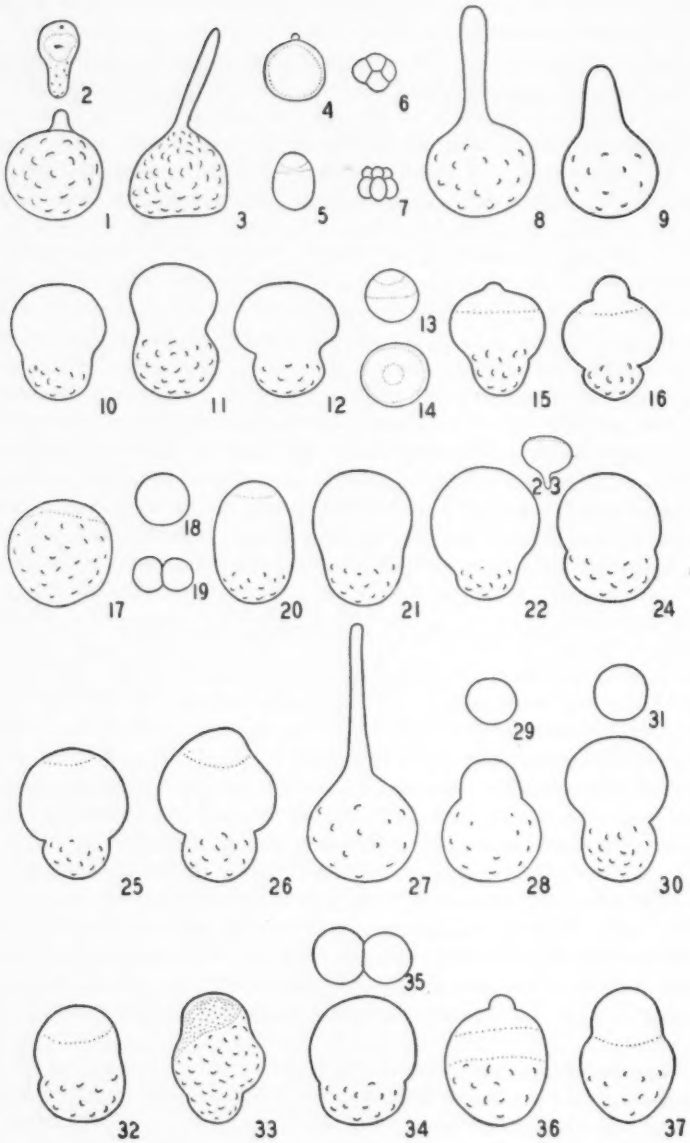
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PLATE I

Polar bodies came off from the tops; none from the bottoms. The tops divided after three hours and 45 minutes, when the controls were going into four cells. The bottoms, with no polar bodies or nuclei, elongated and formed a lobe-like bulge (Plate I, 10–12) in three hours and 42 minutes which partially returned in an hour. Later the bottoms assumed the shape drawn in Plate I, 15–16, with a lobe-like antipolar portion and a clear region at the opposite end with a nipple-like protrusion. No division took place.

In another set centrifuged for 8 minutes at more than 3,000 r.p.m. the tops were small and clear (Plate I, 18). The oil had been driven out of them. Two of these divided later (Plate I, 19) into two and then into four cells. The bottoms were relatively large and produced lobes (Plate I, 20–21).

Newly laid eggs were centrifuged one hour and 20 minutes before the second lobe appeared. The tops were few and small, Plate I, 23; the bottoms conspicuously large, Plate I, 22; and, as the sequel showed, half of them retained the nuclei, presumably both egg- and sperm-pronuclei. When removed it was evident that some protoplasm had remained with the bottom. All the bottoms developed a lobe, which later retreated both in those with and those without nuclei. In these bottoms, presumably the ones without nuclei, the lobe was less marked. Those without chromatin became spherical, and after two hours developed good lobes, Plate I, 24. Fifty minutes later they were spherical again, and an hour later lobes again appeared (Plate I, 25–26). Once more they became spherical, but were not further observed. It is obvious that when more of the protoplasm had been left in the bottoms they underwent a succession of lobe formations more conspicuously than when more of the protoplasm was removed. Stained preparations showed no elimination of chromosomes (i.e., polar bodies) in these bottoms.

In another set the tops were not found at the surface, but were in the outer end of the tube mixed with the bottom, and attached by thin threads to the yolk portion. The tops were no doubt about to separate. Some of the bottoms had long protoplasmic extensions (Plate I, 27) which retracted later. As the subsequent development showed, some of the bottoms segmented normally, although—and this is significant—they did not give off polar bodies. In the normal control, polar lobes were present one hour and 17 minutes after the other eggs had been centrifuged. The relative size of the tops and bottoms are shown in Plate I, 28–31. Of the tops, 7 divided, 11 did not; and of the bottoms, 8 did not divide and 17 did so. This gives approximately the ratio of the bottoms that retained the sperm-nucleus and those that

did not. The bottoms that did not divide had lobes (Plate I, 32) present two hours later. At this time some of them showed a conspicuous accumulation of protoplasm at the polar end; others were spherical with a protoplasmic cap. The lobes of the bottoms slowly retracted; the bottoms remained in a spherical form for one hour, and after 25 minutes small lobes appeared on two of them but not on a third. Stained preparations (Plate I, 33) showed no chromatin present in these bottoms. The centrifuged eggs that had divided were then in the resting two-cell stage.

At the same time that the last set was centrifuged, eggs from the next capsules in series were rotated in the opposite arm of the centrifuge. The second lobe formed in these at about the same time as in the former. Many tops were present, and most of the bottoms were flattened and disintegrating (absorbing water). The few that remained are described below. They were smaller than the whole egg and developed lobes at the same time as the controls (one hour and 40 minutes after centrifuging). Forty-three minutes later they became spherical, and 40 minutes later lobes reappeared. Forty-five minutes later they were again spherical (one in division). Two of the tops divided at the same time, and 8 did not. Later, 5 tops were in four-cell and 4 had not divided, although polar bodies were on them, meaning that the egg-nucleus was present, but the sperm-nucleus had gone off with the oil. Forty minutes later the bottoms were spherical.

Several other sets were followed, but gave no results differing from the preceding ones. In some of the sets, 325 gm. sugar to one liter water was used; in others 374 gm. sugar. In general, when the bottoms divided, the tops did not, whether large or small, which means that the chromatin had remained in the bottoms and presumably the male pronucleus also.

A set of eggs was centrifuged for 5 minutes at 2,680 r.p.m. In the control the first polar body came off one hour and 27 minutes later. There were many tops. The bottoms had a protoplasmic extension when removed. About half of the bottoms gave off polar bodies. Lobes were present in the bottoms two hours and 36 minutes after centrifuging, and 30 minutes later some of them divided. The bottoms that did not divide had good lobes (Plate I, 34). One hour and 13 minutes later 8 tops had not divided and 7 had divided (Plate I, 35) meaning that the sperm-nucleus was absent in half of them. Of the bottoms, 31 divided (some with polar lobes), others not. Later, the latter became spherical; 3 tops divided into four-cell; 3 did not divide. An hour later the undivided bottoms had a protoplasmic zone (Plate I, 36–37) with a nipple-like protoplasmic extension, and an hour later,

when killed, a broad zone of protoplasm, which, as shown in stained preparations, did not contain any chromatin.

Centrifuging in Raffinose Solutions

In all later experiments a solution of raffinose was used in place of the cane sugar solution. At first 3.5 gm. of raffinose to 25 cc. tap water was used. Later 7 gm. was used, which gave better results. After dissolving the raffinose on a water bath at 45° C. it made a thick syrup. The eggs were added with a little sea water at the top of the small centrifuge tube filled with raffinose. In the 3.5 gm. and 5 gm. solutions the eggs sank to the bottom; in the 7 gm. solution they stayed at the top, but were driven to the bottom on centrifuging. The stronger solutions gave larger tops. Nearly all of these sets were centrifuged as soon as laid. After 10 minutes centrifuging at 2,500 r.p.m. the tops were floating near the surface or just below it. The bottoms were in the centrifugal end of the tube, and generally flattened against the glass. The tops were often as large as, or even larger than, the bottoms. There were individual differences in different sets due to the stage of the eggs when laid, and probably also to differences in the viscosity of the eggs of different individuals.

Eggs were centrifuged for 10 minutes at 2,500 r.p.m. and gave many rather small elongated tops that rounded up later (Plate II, 3). The second lobe developed after two hours in the control, and also in the large bottoms (Plate II, 2) but no bottoms showed polar bodies. An hour and a half later lobes appeared again (Plate II, 4). Half of these bottoms (16) cleaved, and half remained spherical without dividing. Twenty minutes later lobes were present on the bottoms that had not divided. Some of the tops had divided. Twenty-four bottoms in all cleaved. It is clear here that the pronuclei had not been driven off into the tops in most cases. It is also evident that, since no polar bodies came off either in those that divided later or in those that did not, the chromosome groups in one or the other kind of bottoms must have been tetraploid or pentaploid. The bottoms that divided presumably had both egg nucleus and sperm pronucleus.

In the following experiments a different solution of raffinose was used. To twelve drops of raffinose (7.5 grams of raffinose to 25 cc. of tap water) were added three drops of tap water. Experience showed that this mixture gave more and better tops.

When removed from the centrifuge many of the tops from the raffinose, like those from cane sugar, are elongated with pointed ends, but some of the tops are more rounded. Later, in sea water, they become spherical, or nearly so. Sometimes the middle of the elongating

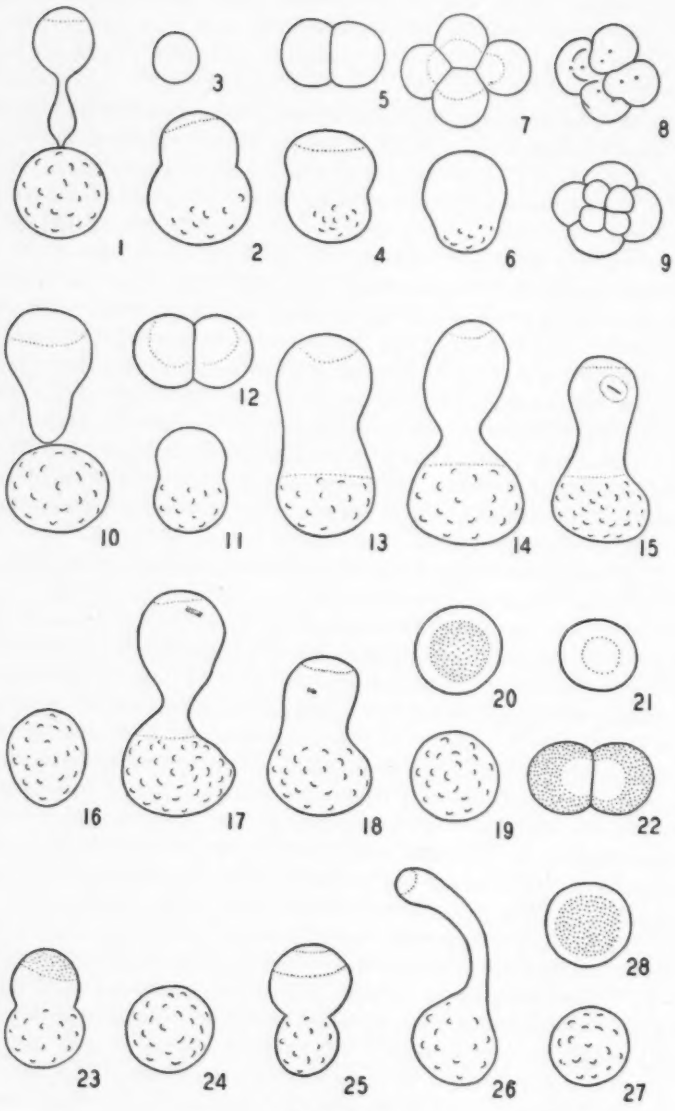

PLATE II

middle part of the egg also constricts off after the tops have separated, giving pieces of various sizes here spoken of as "middles." They contain some or all of the protoplasmic part of the egg. These also round up later. They contain no oil and often no nucleus, which goes with the tops. The tops contain all or part of the oil, and the more deeply stained protoplasm in which the chromosomes are frequently present. Rarely the sperm-nucleus can be identified in the tops. Generally it cannot be found. Since many of the tops divide later, there can be no doubt that the sperm-nucleus is in them. Some of the oil may be driven out of the top. A globule of oil is seen not infrequently outside the apex of the top. Of the many sets centrifuged in raffinose only a few typical examples need be described.

Eggs centrifuged for 10 minutes in raffinose at 2,500 r.p.m., thirty minutes before the second lobe, gave off tops that were nearly the same size as the bottoms (Plate II, 5–9). Some tops were not yet off when removed from the machine. Some of the tops divided later into two cells. Some of the larger bottoms that retained protoplasm and chromatin, but gave off no polar bodies, became lobed (Plate II, 6); others that were small without protoplasm remained spherical at this time. At the eight-cell stage the tops gave off micromeres, apparently in a dextrospiral (Plate II, 8–9). The small bottoms, when stained, were found to contain no protoplasm or chromatin at the apical end.

Another set of eggs was centrifuged for 10 minutes at 2,500 r.p.m. in raffinose. When removed, many of the tops were separating (Plate II, 10). Some of the tops were as big as the bottoms. Later 16 tops divided into two cells (Plate II, 12), and 8 did not divide. The bottoms were constricted in the middle at this time (Plate II, 11). Each had a slightly granular apical end. Stained preparations showed that there was no visible chromatin in the bottoms. The dividing tops (18) went into four equal cells and then into eight cells with micromeres. No lobes appeared in the tops. Ten did not divide. After 2 hours and 45 minutes the bottoms still showed the constrictions, i.e., they had not rounded up.

Another set, centrifuged for 5 minutes at 2,500 r.p.m., gave some good tops, but the separation had not been complete in some of the bottoms as seen alive (Plate II, 13–14). These show well the method of constricting the upper from the lower half of the egg. When preserved (Plate II, 15–18) a plate of chromosomes was found near the end of the clear protoplasm and beneath the oil.

Eggs centrifuged for 15 minutes at 2,500 r.p.m. gave nearly equal tops and bottoms. Nine tops went into two cells after 3 hours and fifteen minutes; 16 bottoms became constricted; others not. Thirty-

five minutes later 12 tops were in two cells, 33 had not divided; 12 bottoms were constricted, 46 not. Forty minutes later 2 tops were in 4 cells; 16 bottoms were constricted; the rest were spherical. Twenty-one minutes later, 12 bottoms were constricted, the rest spherical, and 3 tops were in the four-cell stage. Thirty minutes later, 3 bottoms were slightly constricted, the rest spherical; the tops were in four cells. Nineteen minutes later 2 tops were in eight cells; one was still in two cells; the bottoms were as before.

Eggs centrifuged for 10 minutes gave large tops that were about the same size as the bottoms (Plate II, 19–21). A few bottoms had not pinched off from the tops (Plate II, 23, 25, 26). One and a half hours later, 20 tops had polar bodies; the larger bottoms (without polar bodies) had lobes; 16 middles had no oil and no polar bodies; 22 small bottoms were spherical. Two and one-half hours later 13 tops were in two cells (Plate II, 22), and the bottoms were constricted; the latter showed some protoplasmic material at the apical end. An hour later the bottoms were still constricted and, after another 30 minutes, 20 tops were in four cells; 8 big ones had not divided; one middle had divided into three cells; the smaller middles had not divided. At this time 5 bottoms were spherical and 2 were constricted.

Centrifuged in Sea Water

Newly laid eggs were taken from the capsule and centrifuged in sea water at 2,500 r.p.m. for 5 minutes, and again for 4 minutes. They were flattened on the bottom and stratified, but the tops did not come off. The yolk burst when an attempt was made to dislodge the eggs. Another lot was centrifuged at more than 3,000 r.p.m. for 10 minutes; the eggs were stuck to the glass and could not be removed even after an hour. No tops had come off.

Centrifuged Inside the Capsule

Inside the capsule the eggs are imbedded in a sticky jelly. When centrifuged in the capsule the eggs collect in a plate of cells, and if crowded become columnar in shape. The oil comes off as small globules that do not contain chromatin. They float to the end of the capsule opposite the mass of eggs and, when the capsule is opened, to the top of the water. None of these oil-tops divide.

Newly laid eggs were centrifuged inside the capsule for 12 minutes at about 3,000 r.p.m. The capsule was opened under picrosulphuric acid. Most of the bottoms were stuck to the wall and came off in a lump. The stained bottoms had a clear outer end (stained blue). The

chromatin was in this region. This "middle" part, if pinched off or broken off, contained a metaphase spindle.

Other similar eggs were centrifuged for 10 minutes at more than 3,000 r.p.m. All were in a lump at the bottom of the capsule, arranged in a palisade, with inner pointed clear ends. Sixty oil-tops were counted. The capsule was opened after 1½ hours. Many large middles were found which an hour later divided into two equal cells. Some of the bottoms did not divide; others divided.

Eggs were centrifuged in the capsule for 10 minutes at 2,980 r.p.m., and then, since only some of the oil-tops were off, for 5 minutes more. All of the oil-tops were off, i.e., that region from which the normal polar bodies come, and the bottoms were flattened against the capsule, and against each other. After about an hour the capsule was opened. After another hour nearly all the middles were pinching off (Plate III, 1–5). Each contained a little oil, the rest was clear protoplasm. Seven minutes later these were killed and stained. Except for one middle, no polar bodies were present, but a large nucleus, or two or three smaller ones, was present in the protoplasm (Plate III, 1–4), or in the neck of the protoplasm. These late-developing middles might be regarded as polar bodies, but if so it is only because some of the chromatin at the time when the polar bodies are due to develop happens to lie within the protrusion of protoplasm that may or may not pinch off. In fact, all the chromatin may come to lie in the protrusion as a large vesicle. It is probable that when these bottoms were killed the first division was about to come on.

Eggs were centrifuged for 10 minutes at 2,500 r.p.m. The oil tops were off most of the eggs. One hour and twenty minutes later the eggs showed the second lobe; some had one polar body out (Plate III, 10); others did not show any polar body (Plate III, 9), and others had a clear middle still attached (Plate III, 6–8). An hour and 23 minutes later the eggs were about to divide (Plate III, 6–7). The yolk lobe was constricted. Some eggs had large middles attached. Eighteen minutes later the eggs were about to divide, and were killed and stained. Some of the eggs divided regularly into two cells with a lobe: one had two polar bodies; one egg had a dividing nucleus, half in the middle, half in the bottom. If this is a cleavage figure, the middle is a blastomere rather than a polar body. Another divided egg had a middle (with a faint mass of chromatin) which might possibly be called a polar body.

Eggs were centrifuged for ten minutes at 2,980 r.p.m. The oil-tops came off. The bottoms were compressed into hexagons against the wall of the capsule. When they had rounded somewhat the capsule was opened (one hour and 13 minutes later). No polar bodies were seen

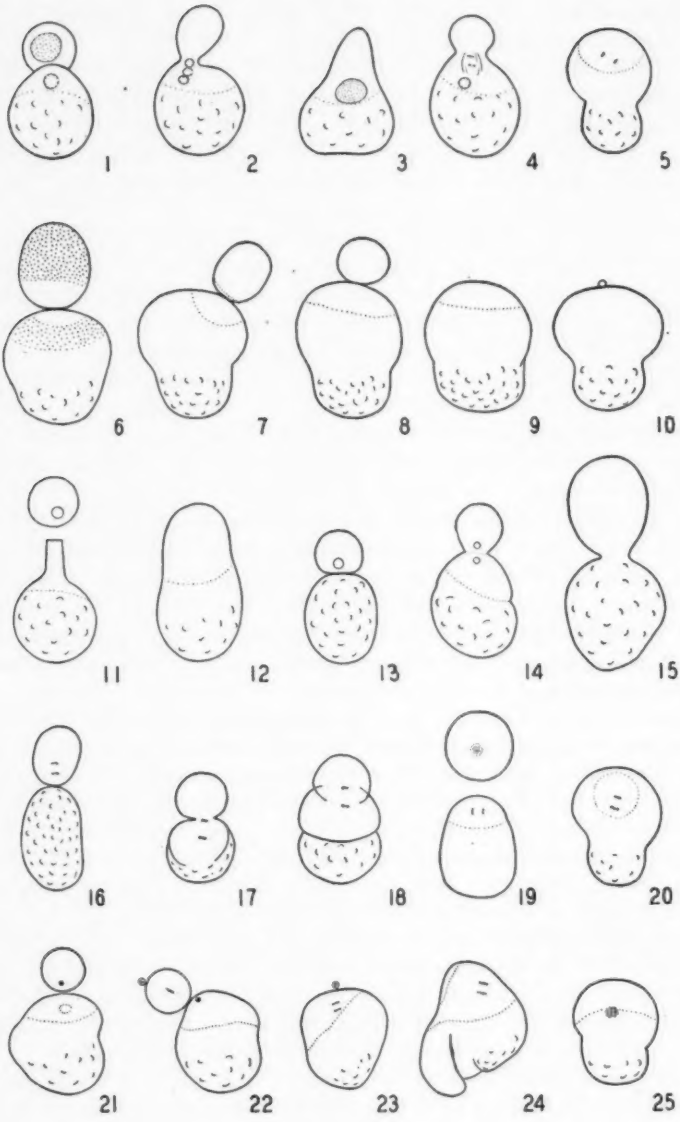

PLATE III

except in two eggs. Several middles constricted off (Plate III, 11–15). Each contained a nucleus (Plate III, 11, 13, 15). In some of the bottoms a clear middle was still partly constricted off and may have contained a nucleus in only the clear part. Possibly these middles, too, might be interpreted as polar bodies.

Centrifuged for 10 minutes at more than 3,000 r.p.m. many eggs after two hours constricted off the middles (Plate III, 16–20). Some middles were entirely free, others were attached. The second lobe was present in eggs killed two hours after centrifuging (Plate III, 20). No polar bodies were given off, but the second anaphase spindle was present in most eggs. Metaphase spindles were present either in the middles (Plate III, 16), or in the constriction between the middle and the bottom (Plate III, 17–18), or in the bottom (Plate III, 19). Whether these middles should be called polar bodies seems doubtful.

In one set centrifuged for 20 minutes at 2,680 r.p.m. the eggs were compressed into three clumps against the capsule. An hour later they had separated somewhat, and two hours later were removed from the capsule, killed, and stained. In all of them the second lobe was present. The condition of the eggs is shown in Plate III, 21–25. Most eggs had no free polar bodies. Several had attached middles. Two of these had chromatin in them. The second spindle, in anaphase, was present in some of the eggs. One egg had a first polar body attached to the middle (Plate III, 22). In another it was attached to the egg (Plate III, 23) and below it an anaphase spindle.

Conclusions

The preceding experiments, concerning the development of the second and third lobes in eggs from which the tops or middles have been centrifuged off, may be compared with the rhythmic behavior of the isolated third lobe described in a preceding paper (1933). There are several categories of cases. More or less of the protoplasm may be present in the tops or in the middles. Accordingly, the sperm-nucleus and the egg-nucleus may be both removed from the bottoms. Again, only the sperm-nucleus may be removed and the chromatin of the egg-nucleus may remain in the bottom. This chromatin may consist of only the reduced egg-chromatin (if the polar bodies have been given off), or the egg-chromatin may have divided in the egg without being reduced by the extrusion of the polar bodies (giving a triploid, tetraploid or pentaploid group). There is the further possible case, viz., the egg-chromatin being thrown out in the top while the sperm-nucleus remains in the bottom.

The evidence concerning the lobe is explicit. The second lobe develops, whether or not polar bodies are extruded, in the presence of the egg-chromatin alone. Furthermore, the evidence shows that the second lobe develops even when all the chromatin has been thrown out of the bottom. It is true the lobe appears less well developed when all the chromatin is absent, but even then it appears and its less conspicuous formation may depend on the smaller size of the bottom.

The results also show that there is a rhythmic series of changes in the bottoms. The second lobe is withdrawn and the bottom is spherical again; then the lobe appears at approximately the same time (or is somewhat delayed) when the third lobe develops in the control. The alternate appearance and disappearance of the lobe has not been followed so far or in as much detail as in the cases described briefly in my former paper, in which the isolated third lobe only was studied. The latter situation appears in some respects a more interesting case, since it is the next to the last stage when the yolk lobe appears.

Discussion

Since most of the problems connected with the experiments described here have been mentioned in the text, it does not seem necessary to go over the ground again. A few points may, however, be mentioned that involve comparisons between the behavior of the separated lobe-end of the egg (the "bottoms") obtained by centrifuging and those obtained by isolating the third lobe after it is constricted from the egg. Since the former are obtained before the polar-bodies are set free, and the latter after the first cleavage is completed, it is possible that the antipolar region may have undergone certain progressive changes during the interval. In fact, the behavior of the two regions is somewhat different. For instance: when the third lobe is shaken off, it undergoes a series of rhythmic changes described in the preceding paper. Wilson, who first observed the same phenomenon in the isolated lobe of *Dentalium*, suggested that the isolated lobe formed a lobe on itself. I have pointed out that since the isolated lobe may continue to repeat this several times (more times than does the normal egg), it is possible that the constrictions do not represent so much the repetition of lobe formation as they do the change that this part of the egg undergoes after it becomes incorporated in the *D* blastomere.

When the rhythmic changes in form of the isolated third lobe are compared with the changes in shape of the non-nucleated bottoms obtained by centrifuging, it is clear that the bottoms undergo rhythmic changes that resemble to some extent those of the isolated third lobe.

But there are differences, and in fact such would be expected, since the bottom might (or might not?) be expected to form a lobe three times in succession, each of different character from the others. If the first lobe formed in such bottoms it might pass unnoticed. It was not observed. If the second lobe formed, it should be quite conspicuous, but like the normal second it would not be expected to pinch off. If the third lobe formed it might be expected to completely pinch off. Nothing of the sort was observed. On the contrary, the succession of changes in the bottoms is not so conspicuous, and perhaps not so prolonged as those of the isolated third lobe.

A comparison should also take into account the possibility of progressive changes in the whole egg during and after polar body formation. In the absence of the polar hemisphere in the bottoms the changes that normally occur in that hemisphere may be restricted or lessened, while these changes may have happened by the time the third lobe is formed; hence comparisons would scarcely be worth making.

The fact that the lobe forms when its interior material has been driven out shows that the formation of the lobe is not dependent on its interior materials. Since it develops approximately at the same time as does the lobe in the whole egg, namely, when the mitotic figure has reached a certain phase, and when the mitotic figure may be in a part of the egg (at the side, or even in the antipolar hemisphere itself) different from its position in the normal egg, it follows that lobe formation, as such, is not a by-product of the mitotic figure, but that the development of the two represent synchronous and more or less independent changes in the egg. Since the interior of the lobe in the inverted eggs may be composed in part of oil and in part of protoplasm it seems to follow that it is in the surface layers, not disturbed by centrifuging, that the cause of the phenomenon is to be sought. This statement is not in conflict with the fact that the complete pinching off of the third lobe is due in part to the cleaving of the polar hemisphere ino two equal parts, leaving the lobe outside (see below). The more complete isolation of the lobe region at the time of the first cleavage is due to the rounding up of the blastomeres, which is a phenomenon superimposed on the lobe-formation.

The cleavage of the tops, both small and large, into two equal parts, then into four equal parts, and then into the unequal micromere division, invites comparison with the results obtained with fragments of the eggs of *Chaetopterus* described by Wilson and by Whitaker and Morgan. In *Chaetopterus* the first division of the fragment is unequal, as it is in the normal egg, although the polar lobe of this egg is very small in comparison with that of *Ilyanassa*. Evidently the inequality in the *Chae-*

topterus egg is not due to the presence of an antipolar lobe region through which the first division can not pass. In *Ilyanassa,* on the contrary, the first cleavage of the normal egg is into equal parts in the polar hemisphere, and gives the impression of passing to one side of the antipolar region as though the cleavage plane could not pass through it; but the equal division of the top shows that the inequality in *Ilyanassa* is not a primary inequality as in *Chaetopterus,* but on the contrary the division is equal or nearly so. The subsequent fusion of the lobe with one blastomere is responsible for the apparent inequality of this division.

In both animals fragments of the polar hemisphere do not form lobes, while the antipolar hemispheres do produce lobes. In this connection a further question arises. Is the first division across the antero-posterior axis of these eggs? Does such a condition pre-exist in fact, or develop only during or after the division? I do not know of any decisive answer to this question. Even in the eggs of the frog, of *Amphioxus,* or of *Styella* it has not been convincingly proven, in my opinion, that an antero-posterior axis exists prior to fertilization. After that event it certainly appears to be present. So important is the decision on this point that I shall remain skeptical until more convincing evidence is produced. If it is true that such an axis exists, its presence would seem to simplify some of our difficulties of interpretation, but the wish to have the question settled should not affect our attitude in regard to the evidence.

So far as the location of the lobe before fertilization is concerned, it might appear that, if there is a preformed antero-posterior structure to the egg, the incorporation of the lobe into one only of the first two blastomeres could be explained as due to some peculiarity of the surface layer on one side that prevents one blastomere from completely cutting off—hence the lobe remains as part of one blastomere (the dorsal one) only. But attractive as such an assumption might appear, it is also possible that just before cleavage an antero-posterior change takes place on one side of the egg which is responsible for the failure of the lobe to pinch off on that side. The whole question of the relation of the sperm entrance and the plane of fusion of the pronuclei is concerned in this question; and it is better, I think, not to prejudge it, even though it may seem to offer a simple way out of a difficult problem to assume that a preformed dorsal side pre-exists. If, as has been shown, there is a definite relation between the entrance point or penetration path of the sperm and the establishment of an antero-posterior axis, then to assume a preformed axis to solve formally one problem may land one in a contradiction with respect to the

other problem. If, however, the antero-posterior difference comes in after fertilization, both problems may find a common solution.

Towards the end of the experiment several cases were observed in which the first or second polar spindle lay at the base of a protoplasmic protrusion of the bottom (Plate III, 17, 18, 14). Into this protrusion one or more polar nuclei may pass. The protrusion may later pinch off. It might then be called, with reservations, a giant polar body and compared with a somewhat similar process Conklin has described for *Crepidula*. Without a fuller series of observations on *Ilyanassa* I do not feel like deciding whether the isolated "middles" are comparable to polar body formation, or whether there is something here *sui generis* owing to the very unusual conditions that are present at the time.

THE RHYTHMIC CHANGES IN FORM OF THE ISOLATED ANTIPOLAR LOBE OF ILYANASSA

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In a previous paper (1933) I have described the rhythmic changes in shape of the third yolk lobe when isolated. In the summer of 1934 I made at Woods Hole the additional observations described below.

The simplest way to separate the third lobe from the two blastomeres is to squirt the egg gently in and out of a pipette at the time when the lobe has pinched off from the two cells. It often comes off when the eggs are removed from the capsule if they are in the two-cell stage. It is noticeable that it is less often injured than are the two blastomeres which, when shaken off in the two-cell stage, often go to pieces at once. The isolated lobe soon becomes spherical. Sometimes, however, it too absorbs water, swells up and dies.

The following record gives the history of the isolated third lobes. It is not easy to give an account of the change that takes place in the isolated lobes without going into great detail. The lobes were followed continuously for several hours and sketches made. I have selected four records out of twelve, which tell sufficiently the story with the aid of the drawings.

Case 1

The two-cell stage was shaken at 12:05 P.M.; at 2:25 the isolated lobes were oval (Figs. 1, 2); at 2:50 pointed or with a nipple (Figs. 3, 4). They were round at 3:13 (Fig. 5), blunt at 3:50, pear-shaped at 4:08 (Figs. 6, 7, 8); more constricted at 4:17 (Figs. 9, 10, 11), and at 4:37; round at 4:53 (Fig. 12); round or elongated at 5:37 (Fig. 15); constricted at 5:45 (Figs. 14, 15, 16).

Summary: The lobe became oval or pointed when the controls were in 4-cell stages, and constricted again when the controls were in 16-cell stages. They then became round, and again constricted. There is no obvious correlation between the times of cleavage of the control and the alternate phases of constriction of the lobes.

Case 2

The 2-cell stage was broken apart by squirting in a pipette. The lobes were slightly pointed at 12:14 (Fig. 17); the lobes rounded up at

12:20, were oval at 12:38 (Fig. 18), and at 12:52 to 2:40 they were round (Fig. 19). At 3:05 to 3:21 (Figs. 20, 21, 22, 23, 24) they became pointed, then constricted (the whole eggs were going into 8-cell stages). At 3:40 they were round (Fig. 25) until 4:15 to 4:43 when they became constricted (Figs. 26–32). At 5:15 the lobes were spherical. Summary: Here the lobes became constricted twice, much more slowly than the cleavages of the normal control.

Case 9

Lobes were shaken off at 11:55. They were round at 12:03 and remained so until 1:53. At 2:56 (Fig. 33) they became constricted (the normals were in the 4-cell stage). At 3:48 the lobes were oval (Fig. 34), one was round; at 4:14 they were pear-shaped or slightly constricted (Figs. 35, 36); at 4:45 they were spherical (Fig. 37); at 5:24 elongated (Fig. 38) with clear ends. At 6:22 they were oval or round (Figs. 39, 40); at 7:13 to 7:55 (Figs. 41, 42) they had clear ends with a constriction. At 8:52 to 9:18 they were round; at 8:52 they were still round, and at 9:50 (Figs. 43, 44) they were oval or with a slight constriction. Summary: In this case there was a succession of changes in shape and an accumulation of clear protoplasm at one end. The changes were not obviously synchronous with the changes in the cleaving eggs.

Case 11

Shaken off at 2:35, the lobes were round at 2:55. At 3:35 some were oval with a clear nipple (Fig. 45); others pear-shaped. At 3:46 they were round (Fig. 46); at 4:11 to 4:42 most of the lobes were oval with clear ends (Fig. 47); at 5:22 they were round again (Fig. 48); at 6:19 they were pear-shaped (Fig. 49); at 7:11 they were round (Fig. 50). At 7:50 they were oval and elongated (Figs. 51, 52); at 8:22 they were round; at 9:15 most were round (Fig. 54), some with a clear end (Fig. 55) which was still present at 9:53 (Fig. 56).

The question now arises as to whether these changes in the form of the lobes is to be interpreted to mean that the isolated lobe continues alternately to form a succession of lobes, or whether the changes in shape represent some other kind of rhythmic activity. I have discussed this question in my previous paper and raised there certain theoretical objections to the literal interpretation of the changes in shape as repetitions of lobe-formation, chiefly because these isolated third lobes may go on alternately constricting and rounding up more times than this region does when it is a part of the normal egg.

The experiments, reported in other papers in which the egg is constricted into two parts by centrifuging in raffinose, reopen the question of the interpretation of the rhythmic changes in the "lower" half (antipolar hemisphere) of the eggs there spoken of as the bottoms. These experiments show clearly that the "upper" polar half of the egg,

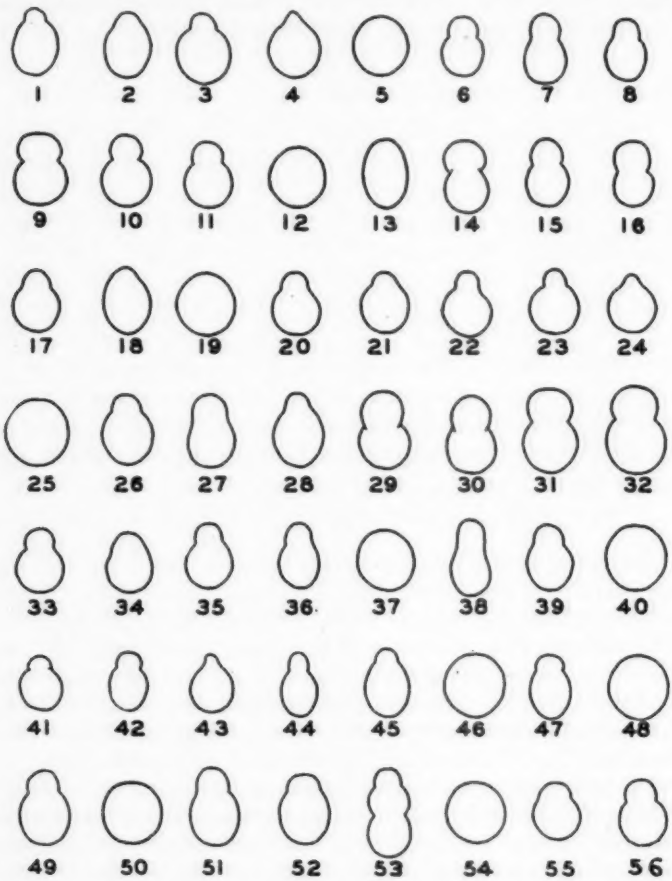

PLATE I

that does not contain the lobe-forming portion of the whole egg, does not repeat the type of cleavage of the whole egg since no lobe appears on it, either before or after the first cleavage. Again, it is clear that the antipolar half or bottom, which contains all of the lobe region,

develops a constriction at least twice, whether it contains the egg-chromatin, or whether none of it is present. Since these two changes are vaguely synchronous with the lobe-formation of the whole egg, they appear to correspond to that phase. If this is correct, it reopens the discussion of the changes in shape of the isolated third lobe.

That the changes in the third lobe are not strictly synchronous with the second cleavage is apparent—they are much delayed. This in itself might not rule out the interpretation that they represent lobes, but it is to be remembered that the lobe appears only once more after the first cleavage ends, while the isolated third lobes come and go at least three times. It should also be remembered that, strictly speaking, the change in shape does not give a reduced picture of the whole egg and its lobe, as a comparison of the figures illustrates. Furthermore, after the second cleavage, the antipolar hemisphere normally becomes incorporated in one of the first four cells, and at the next cleavage this cell pinches off a micromere from the apical end. The changes in shape of the isolated third lobe resemble somewhat more this process of constricting off micromeres than they resemble the lobe-formation of the early stages. I am inclined, therefore, towards the same interpretation of the later rhythmic changes of the isolated third lobe, rather than the interpretation that lobe-formation repeats itself several times. There is also a general consideration that should be given some weight. The cleavage stages are progressive steps. Even isolated blastomeres carry out the partial pattern rather than returning to the previous stage. Hence I think we might expect the rhythmic changes of the third lobe to do this also. If so, the lobe-constrictions and concentration of clear material at the polar end correspond in a way to the later development that this part normally undergoes if it remains in the egg.

Returning again to the centrifuging experiments, the behavior of the bottoms may be interpreted to mean that they represent the two normal stages of lobe formation. The later phases that appear would then be interpreted in the same way as those of the isolated third lobe. There is, then, no inconsistency in interpreting the bottoms obtained by centrifuging eggs in an early phase and the interpretation of the rhythmic changes of an isolated third lobe as representing later stages of development, since the latter begin at a later period. The interesting fact to be emphasized is that this part of the egg deprived of a nucleus continues to undergo rhythmic changes resembling more or less the changes that it normally undergoes as part of the whole egg. In other words: there resides in the protoplasm of the mature egg the property of taking part in the progressive series of changes of the egg independently, to some extent, of influences depending on the activity of the genes at that time, or the presence of mitotic developments.

MODIFICATION OF MAMMALIAN SEXUAL CYCLES

II. Effects upon Young Male Ferrets (Putorius vulgaris) of Constant Eight and One-half Hour Days and of Six Hours of Illumination after Dark, Between November and June[1]

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Introduction

Some experiments with young male ferrets in their first autumn, with six hours of electric light added after dark, led to some increase of spermatogenic activity, to complete activation of testis interstitial cells and of secondary sex-organs and to increased libido, leading to sterile matings on December 10, December 12, and December 21 with females brought into œstrus by similar increases of daily light exposures beginning on October 12 (Bissonnette, 1932). They did not give a clear picture of the differences between the sex-organs of such males on increased lighting and those of similar animals on a constant reduced day.

The rather preliminary study to be reported here was an attempt to test the effects of a constant 8½ hour day in autumn and winter as contrasted with those of "long days" consisting of normal days for the seasons to which 6 hours of electric lighting from an incandescent bulb was added after dark each night, in order to learn whether either of these alterations in day-length modifies the sexual cycles of such young males and, if so, to what degrees.

Material and Methods

On November 10, 1932, six males in their first year were placed in separate cages in two vertical rows, 1, 3, 5, in one series and 2, 4, 6, in the other, facing the light from three large windows of a basement room at a distance of about 7–8 feet.

The left-hand series (1, 3, 5) also faced a 300-watt clear bulb at such distances that the nestbox in cage 1 received light varying from

[1] Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council, 1932–33. Library and laboratory facilities were also furnished by the Marine Biological Laboratory at Woods Hole, Mass.

16.86 foot candles, nearest the bulb, to 9.25 at the back of the box; cage 3 received 16.31 to 8.81 foot candles; and cage 5, from 12.88 to 7.17 foot candles. Light from the windows was not measured, but fluctuated from day to day and with the season. The animals in these cages received decreasing daily daylight periods till December 21 and increasing ones thereafter till June 9. The decrease was about nine-tenths of an hour from 10 hours on November 10 to 9.1 hours on December 21. The increase was about 1.9 hours to February 24 and about 6.1 hours to June 9, when the last two experimentally lighted males were sacrificed. They were also subject to change in daylight intensity partly due to change in the height of the sun above the southern horizon during the day, reducing it gradually till December 21 and increasing it thereafter, partly to changes in daily sunshine.

The right-hand series (2, 4, 6) bore the same relations to the windows and to daylight intensity, but they were covered and shielded from light by a heavy carpet from 4.30 P.M. to 8.00 A.M. daily, during part of which time the electric light was shining into the other series of cages. This kept their day-length approximately constant at 8½ hours per day.

All the animals were behind ordinary window glass throughout the experiment and the light bulbs emitted only negligible ultra-violet radiation.

Food for all consisted of pasteurized milk till about May 1, and natural milk with cod-liver oil concentrates added thereafter, to which were added dog-biscuit and very occasional meat scraps, both raw and cooked, throughout the experiment. The physical conditions of the animals throughout were about normal, though one or two developed light attacks of "foot-rot" before the whole milk and C.L.O. concentrates were introduced into the diet. However, this condition had been found in the previous experiments to interfere in no way with normal or induced sexual activity in these animals.

The position of the light bulb (similar to that used before) was such that it shone most horizontally upon the animal in the upper cage, more and more obliquely upon the middle and lower cages respectively, with distances greater in the lower ones. As noted before, this permits the ferret curled up to sleep to shield his head and eyes from light better in the upper cage than in the lower ones. Such shielding of the eyes was correlated with slower rate of activation sometimes, in spite of the shorter distance from the light and consequent greater luminous intensity at the upper levels (Bissonnette, 1932).

The amount of hair over the scrotum, and the conditions and positions of the testes were tested from time to time by palpation and in-

spection. Sexual libido, copulating power, and sperm ejaculations were also tested at various times as œstrous females were available from a parallel experiment on the effects of hoodwinking these animals and using hoods with eyeholes to learn the avenue of reception of the light stimulus to be described elsewhere. These trials began on January 13 and continued till June. On the former date none of the "short day" males showed any interest in copulating or even mauling an œstrous female. Of the "long day" animals, No. 5 made feeble attempts to copulate, No. 1 made very determined attempts but was unsuccessful in becoming "fast" to her; No. 3 succeeded and remained "fast" to her for 1¾ hours that day and again for 1½ hours the next day. These matings resulted in pseudopregnancy only, as there were no sperms in his ejaculations, as determined by microscopical examination of the fluid drawn off from her vestibule immediately after copulation ceased.

At killing, genital tracts, thyroids, and adrenals were fixed in Bouin's fluid and the hypophyses in Zenker's fluid, with formic acid replacing acetic in both solutions. After the usual paraffin technique, testes and epididymides were sectioned at 10 microns, stained in Heidenhain's iron-hematoxylin and eosin, and mounted in balsam. The other endocrine glands were saved with the rest of the genitalia for later study.

KEY FOR TABLE I

Copulations

⊕− = tried feebly and unsuccessfully.
+− = tried forcefully and unsuccessfully.
++ = tried successfully.
−− = did not even try to copulate.

Sperms in matings

−− = sperms absent.
+N = sperms present, inactive, and few.
+A = sperms present, active, and few.
++A = sperms numerous and active.

Position of testis

A = anterior in groin, near winter position.
P = posterior in scrotum, near anus.

Pregnancy or pseudo-pregnancy

−− = no effect noted.
pP = pseudo-pregnant.
P = pregnant.

No. of animal

1, 3, 5 were exposed to added electric light.
2, 4, 6 were restricted to an 8½-hour day.

TABLE I

Data on sexual activity and behavior of "short day" and "long day" males

No. of male	Date	Copulation reaction	Copulation time	Sperms in matings	Testis size	Testis position	Pregnancy or not	Killed. Remarks.
			hours					
1	Nov. 10	—	—	—	—	—	—	Light began
	Jan. 13	⊕−	—	—	large	P	—	———
	" 14	+−	—	—	"	P	—	———
	Feb. 21	+−	—	—	"	P	—	———
	" 22	++	1	—	"	P	—	———
	" 23	−−	—	—	"	P	—	Killed Feb. 24
3	Jan. 13	++	1¾	−−	large	P	pP	———
	" 14	++	1½	−−	"	P	—	———
	Feb. 20	++	2	+A	"	P	P	Female gave young, Apr. 3
	" 21	++	—	++A	"	P	P	Same female
	Mar. 7	++	—	++A	"	P	P	Female died Mar. 31; 11 embryos
	" 11	++	—	++A	"	P	P	———
	" 18	++	—	++A	"	P	P	———
	Apr. 20	++	1¼	—	small	P	pP	Eggs shed before 36 hrs. after finish
	" 28	−−	—	—	"	—	—	———
	June 8	++	1	−−	"	A	—	Killed June 9: in regression

TABLE I—*Continued*

No. of male	Date	Copulation reaction	Copulation time	Sperms in matings	Testis size	Testis position	Pregnancy or not	Killed. Remarks.
			hours					
5	Jan. 13	⊕−	—	—	small	A	—	———
	" 14	−−	—	—	"	A	—	———
	Feb. 20	—	—	—	"	P	—	———
	" 23	++	2¼	—	medium	P	—	———
	Mar. 6	++	—	—	larger	P	—	———
	" 7	⊕−	—	—	"	P	—	———
	" 8	++	—	—	large	P	—	———
	" 11	++	—	—	"	P	—	———
	" 13	++	—	—	"	P	—	———
	" 18	++	—	—	"	P	—	———
	Apr. 28	—	—	—	small	1A, 1P	—	———
	June 8	++	1⁹⁄₆₀	—	"	A	pP	Ovulation 35 hrs. later
	" 8	—	—	—	—	—	—	Killed
2	Jan. 13, 14	−−	—	—	small	A	—	———
	Feb. 20	−−	—	—	"	A	—	———
	Mar. 8	++	—	—	larger	P	—	Killed Mar. 15
4	Jan. 13, 14	−−	—	—	small	A	—	———
	Feb. 20	−−	—	—	"	A	—	Killed Feb. 24
6	Jan. 13, 14	−−	—	—	small	A	—	———
	Feb. 20	⊕−	—	—	large	P	—	Scrotum getting bare
	Mar. 7	++	—	+N	"	P	—	———
	" 12	++	—	++A	"	P	—	———
	" 19	++	—	++A	"	P	P	———
	Apr. 8	—	—	—	—	—	—	Killed

Results and Observations

Changes in Macroscopic Conditions and in Behavior

About two weeks after lighting began the testes of the "long day" animals (1, 3, 5) started to move backward into a position in the scrotum immediately below the anus and to enlarge and become turgid. The hair then thinned out over the testes till, at time of greatest testis size, the scrotum became almost bare. In this No. 5 lagged considerably behind the other two. In one of the "short day" males this was not delayed as much as in No. 5; but in the other two (2, 4) it was greatly retarded.

The results of these tests are summarized in Table I in detail.

Of the "short day" animals, No. 4 was killed for study on February 24 without showing any sexual libido at all; No. 2 showed sexual libido first on March 8, when he succeeded in copulating but probably without sperm ejaculation (a few non-motile sperms were found in the vestibule of the female, probably from a previous mating with another male giving abundant sperms) and was sacrificed for study on March 15 without conclusive evidence of sperm ejaculation; No. 6 first showed libido enough to try to copulate on February 20, unsuccessfully. He first succeeded on March 7 and left a few non-motile sperms in the vestibule of the female. By March 12 and 19 he was able to give abundant motile sperms in matings. He was killed for study on April 18 when his testes were still at the climax of activity.

Of the "long day" animals, No. 3 was most quickly stimulated and copulated without sperm ejaculation as early as January 13 and 14, and with abundant active sperms by February 20 and 24, inducing pregnancy on the twentieth. However, by April 20, his testes were smaller and soft, and a mating on that date was without sperm ejaculation, though it induced ovulation of the female within 36 hours after the finish of copulation and the granulosa cells had already left the eggs which were well down the Fallopian tubes. He was still farther in regression by April 28 and June 8; though on the latter date he copulated, without sperm ejaculation.

No. 1 was somewhat slower to respond and tried hard but unsuccessfully to copulate on January 13; was successful on February 22, but not on the twenty-fourth, when he was sacrificed (no test was made for sperms as the female had already mated with a male giving abundant active sperms).

No. 5 was slowest to respond. He made only feeble attempts to copulate on January 13 and 14; was successful on February 23, March 5, 8, 11, 13, and 15, but always without sperm ejaculation, so far as could be determined by microscopical examination of fluid from the

vestibule of the females after copulation. By April 28, his testes were again small and one forward in the groin. But on June 8 he copulated for an hour and nine minutes with induction of ovulation within 35 hours of the finish of copulation, though his testes were both forward in the groin and small.

These facts indicate that libido is restored before complete potency for copulation, and both before complete spermatogenesis, but that spermatogenesis regresses before libido and potency to copulate. The copulations on June 8 by males No. 3 and No. 5 were normal in duration and in inducing ovulations in females; but ability to ejaculate sperms was already gone even before April 20 and 28, at least in No. 3, the most quickly activated male.

Histological Conditions

The Testis.—On February 24, after 106 days of 8½ hours duration, the testes of male 4 (Fig. 1) showed seminiferous tubules of about 152 μ diameter, an increase from about 79 μ on December 12 at the autumn quiescent condition (Bissonnette, 1932, Fig. 3) or about 77 μ on November 5 (Allanson, 1932), as compared with about 165–179 μ on February 4 and 8 respectively for the normal ferret (Allanson, 1932). Interstitials were increased in number and activity as compared with those in the completely quiescent autumn condition. Intertubular spaces were distended with lymph, which stained slightly with eosin. Within the tubules were Sertoli cells, spermatogonia, and full-grown spermatocytes; but no further generations of germ-cells. A lumen was present in the tubules and the syncytial cytoplasm was rather sharply delimited from that of the individual cells. Alto-

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Explanation of Plate I

All figures are photomicrographs taken in the first instance at 335 diameters and reduced to about 190 diameters in reproduction.

FIG. 1. Testis section from "short day" ferret, on 8½ hours of daylight per day from November 10 to February 24—106 days.

FIG. 2. Testis section from "long day" ferret, on normal day-length with 6 hours of electric light added per night, from November 10 to February 24—106 days.

FIG. 3. Testis section from "short day" ferret on March 15—125 days.

FIG. 4. Testis section from "short day" ferret on April 8—149 days.

FIG. 5. Testis section from "long day" ferret on June 8—210 days.

FIG. 6. Testis section from "long day" ferret on June 9—211 days.

FIG. 7. Epididymis section from "short day" ferret, February 24—106 days.

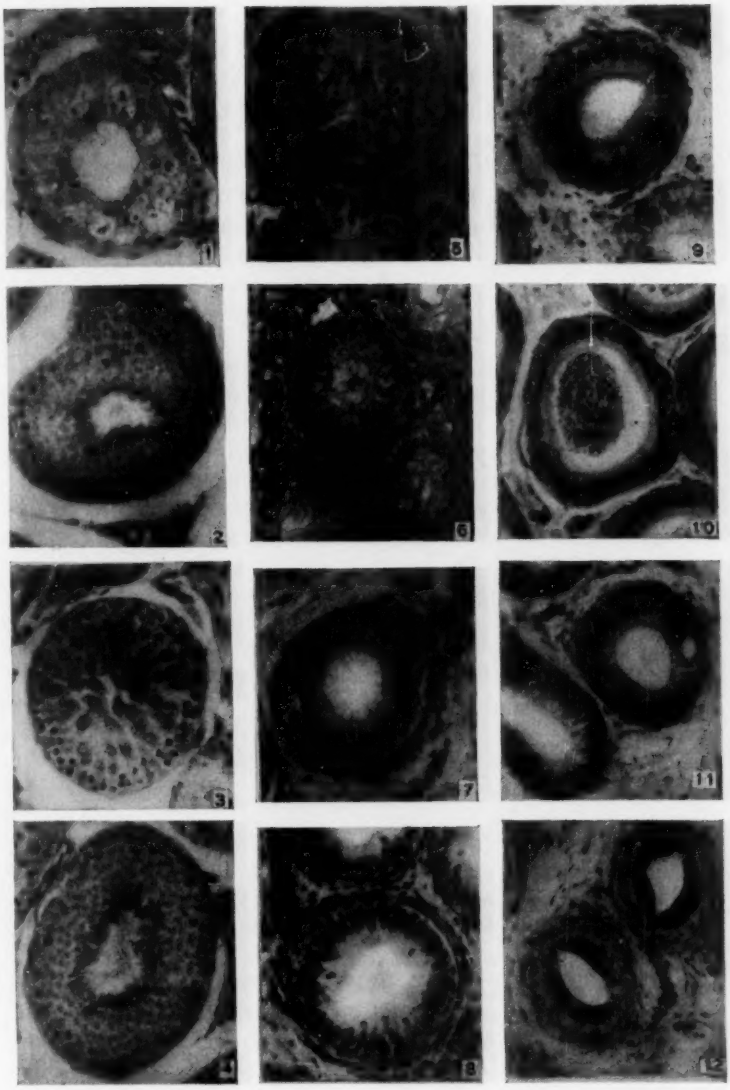
FIG. 8. Epididymis section from "long day" ferret, February 24—106 days.

FIG. 9. Epididymis section from "short day" ferret, March 15—125 days.

FIG. 10. Epididymis section from "short day" ferret, April 8—149 days.

FIG. 11. Epididymis section from "long day" ferret, June 8—210 days.

FIG. 12. Epididymis section from "long day" ferret, June 9—211 days.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12

gether the testes of this animal were definitely retarded in activity by the " short days " as compared with those on the normal light cycle in which spermatogenesis is almost, if not quite, complete by February 4 (Allanson, 1932).

On the same date, after the same period with 6 hours of electric light each night added to normal days, the testes of Male 1 (Fig. 2) were much larger than those just described. Tubule diameters were about 197 μ, even larger than those of ferrets on normal days at March 12 (Allanson, 1932). Interstitial cells were in breeding condition, and intertubular lymph spaces distended with lymph, staining weakly in eosin. Spermatogenesis was complete, and mature sperms were plentiful in the epididymis and vas deferens near the testis (Fig. 8), just as in the animals on normal light cycles on March 2 or 12 (Allanson, 1932).

On March 15th, after 125 " short days," the tubules of the testes of Male 2 (Fig. 3) were about 185 μ in diameter. Interstitials were numerous and active; intertubular lymph spaces large and full. No germ-cells beyond secondary spermatocytes were yet present, except a very few spermatids, not yet showing signs of metamorphosis. Early germ-cell stages were very numerous. This testis had not yet reached the stage normal for February 4 (Allanson, 1932) though its size may not have been retarded.

On April 8, after 149 " short days " the testes of Male 6 (Fig. 4) were in complete reproductive activity in all respects. Sperms were numerous in both testis and epididymis (Fig. 10) and tubules were 176 μ or more in diameter. This male had ejaculated sperms in matings as early as March 7, but not before, so he was also definitely retarded in sexual activation by the " short days." However, the short days did not prevent him from coming into complete sexual activity. Changes in light intensity could account for the activation (Bissonnette, 1933; Marshall and Bowden, 1934).

On June 8, after 210 " long days," the testes of Male 5 (Fig. 5) had passed the climax of activity of germ-cells and were far along in regression. Tubule diameters were only 113–128 μ. However, interstitials were still abundant and apparently active. Lymph spaces were small and not distended. There were no germ-cells beyond synizesis stages of spermatocytes. Lumina were absent in the tubules; cytoplasm was stringy; and many nuclei necrotic. Though this animal was slow to come into activity as compared with the other two " long day " ones, his sex-glands had already long passed their climax and were far into regression, even on a more than usually stimulating light schedule. At this date, ferrets on normal light cycles are still at full activity (Allanson, 1932).

On June 9, after 211 "long days," the testes of Male 3 (Fig. 6), quickest to reach sexual activity, were even farther in regression than those of the preceding animal. Tubule diameters were only 81–99 μ; interstitials were in the typical autumn quiescent condition; and germ-cells reduced to spermatogonia and very few synizesis stages. Only a very few tubules had lumina, and necrotic nuclei were numerous in the stringy cytoplasm.

In both these "long day" animals regression had set in even in spite of a very stimulating schedule of daily light exposures.

The Epididymis (beside the middle of the testis).—On February 24, the epididymis of Male 4 (Fig. 7) had reached almost the completely active condition, with epithelial lining cells about 30 μ tall or more, and the cilia-like processes long and abundant. Sperms were not present. The same regions were at the complete breeding condition in Male 1 ("long day") (Fig. 8), with sperms and cellular debris in the lumen tangled in the long cilia-like processes. Tubules were stretched by contents and the epithelial lining was like that in the preceding "short day" ferret.

On March 15, the epididymis of "short day" Male 2 (Fig. 9) was in breeding condition, with epithelial cells about 42 μ tall, but without sperms. On April 8, that of "short day" Male 6 (Fig. 10) was similar but with sperms in the lumen. Lining was stretched until it was only about 30 μ tall.

On June 8, the epididymis of "long day" ferret No. 5 (Fig. 11) was still in breeding condition, with lining cells 32 μ tall and "cilia" abundant. This condition was correlated with active interstitial cells in the testes; but germ-cells far in regression.

On June 9, the epididymis of "long day" ferret No. 3 (Fig. 12), quickest to respond to light stimulation, was far into regression and almost at the autumn quiescent condition. "Cilia" were short and almost indistinguishable; epithelial lining only 15 μ tall or less, and lacking the clear zone next the lumen of the tubule so characteristic of the active condition of these cells.

Both these "long day" ferrets had undergone regression of the epididymis parallelling that of the interstitials of the testis, but lagging behind that of the germ-cell line, more evident in Male 5 than in the more responsive Male 3.

Discussion

The retardation of testis activity in Males 2 and 4 by the shortened days, in spite of increasing daylight intensity, indicates that increasing day-length stimulates or accelerates onset of spring sexual activity or

that lack of it holds activity back. The much quicker response of Male 6 may be due to more favorable position of the head and eyes for reception of light when curled up to sleep or to greater individual susceptibility to effects by increasing intensity (Bissonnette, 1932, 1933, and unpublished data; Marshall and Bowden, 1934) than in those more delayed.

The slow reaction of Male 5, on "long days" as contrasted with that of 1 and 3, may be due to greater distance from the bulb or to less favorable reception during sleep; but that could hardly account for its being slower than Male 6. Individual idiosyncrasy appears to be a more likely reason until further study and analysis shed more light on the matter.

In spite of these apparently anomalous exceptions, it appears that, for most young ferrets, an increase in day-length leads to acceleration of testis activity, with interstitials preceding germ-cells in effectiveness; and that libido and accessory sex-organs (epididymis) are closely correlated with interstitial cell changes, in agreement with results from previous studies (Bissonnette, 1932, 1933; Marshall and Bowden, 1934; Hill and Parkes, 1933).

On the other hand, again with some exceptions, a relatively constant, short, winter-like day, throughout the spring season of normally increasing day-length, leads to a delay in onset and a slowing up of the spring activity of the testis and epididymis and a late appearance of sexual libido.

Either type of experimental modification of day-length leads to modification of the sexual cycle in such young males by changing the time of onset of libido and sexual power, as related to ejaculation of sperms and spermatogenesis, from the normal one in nature.

Both behavior and histological studies indicate that, even under increasing stimulating light exposures, greater than normal, sexual regression or "anoestrus" (Hill and Parkes, 1933) sets in after a time. Testis activity and libido ("Oestrus," Hill and Parkes, 1933) may be hastened by these increasing exposures to light, but cannot be maintained indefinitely by them (see Bachman, Collip, and Selye, 1934). Regression comes in spite of them and as the result of changes from within the ferrets, presumably from changes of the anterior hypophysis or immunity to its hormones. This is supported by facts brought out by a preliminary casual study of histological conditions of the hypophyses at autopsy throughout these experiments. These indicate an increased activity of the anterior lobe during the progressive changes of the sex-glands and a falling off during regression. This conclusion has been confirmed by Dr. A. E. Severinghaus, who kindly looked over

some of our hypophysis material and agreed with our conclusions. This will be dealt with more fully later.

While regression sets in, in spite of increasing day-lengths, in ferrets, just as in starlings (Bissonnette, 1933, and preceding papers), it is probable that it is hastened by shortening autumn days or that the beginning of the next increase in activity is delayed by the reduction of light. Regression need not depend on a falling light curve but occurs sooner or later even on a rising day-length. How soon regression may be stopped and reversed after it begins or how far destruction of germ-cells must go before the next progressive changes may be induced remains to be seen.

The discovery of Hill and Parkes (1934) that ferrets of unmentioned age, of both sexes, come into "oestrus" in spring, even when subjected to total darkness for 23½ hours or more per day continuously from January 24 onward up to 5½ months, and that a second oestrus may follow after suckling young under these conditions, is very important in this connection. Their conclusion that the increasing length of day in spring is not essential for the appearance of normal oestrus in ferrets in spring, is perhaps too sweeping in its implications and not completely warranted by their published data. "Oestrus," to be normal, must come at the normal time and by the same time sequence as in nature. Their data show that the "oestrus" occurring under their experimental conditions was later than normal and took longer to reach the receptive stage than normal. To be conclusive, their experiment should have begun in November or December, not after their animals had been subject to over a month of increasing light exposures, already known to be sexually stimulating (Bissonnette, 1932, 1933; and their own earlier experiments). This will be discussed more fully in a later paper.

Recent experiments by Allanson, Rowlands, and Parkes (1934), adding light from October 12 onward and following up with injections of pregnancy urine extracts, gave pregnancies from matings on December 29 and January 1, and litters of 9 and 10 young born February 8 and 11 and show that added lighting in autumn increases sexual activity in males as well as females. Some of their males reached complete sexual activity in early January from increased lighting alone. Their litters show that eggs and sperms induced by artificial activation are fertile and viable. Some of our own experiments to be reported later led to complete spermatogenesis and accessory sex-organ activity before November 7, after 36 days of experimental lighting alone. They also showed onset of regression following such activity in spite of increasing light exposures as a result of some internal changes in the animals or

on passing the optimum intensity or duration of daily light periods. To date the internal change seems to offer the best explanation, based upon a time factor (Bachman, Collip and Selye, 1934).

Summary and Conclusions

1. Three immature male ferrets were kept on "short days" from November 10 till killed for study of testes and epididymides on February 24, March 15, and April 8.

2. Three similar males were kept on "long days" from November 10 until February 24, June 8, and 9.

3. Mating reactions and sperm ejaculating power were tested from January 13 onward.

4. Onset of sexual activity was delayed considerably in two of the "short day" animals, as compared with normals, only slightly in the third.

5. It was hastened in two "long day" animals, not in the third, which was late.

6. In both groups, interstitials came into activity before germ-cells, and, in the "long day" animals, remained active longer than germ-cells, when regression followed the climax of activity.

7. Changes in epididymides were more closely correlated with those of interstitials than with those of germ-cells.

8. In "long day" ferrets, quickly activated or not, regression set in well before June 8, at which time males on normal light cycles are at complete sexual activity, in spite of increasing duration and intensity of daily light exposures.

9. Therefore, while sexual activity may be induced or hastened by increasing daily light in young male ferrets, as in females, in these males regression sets in after a time even in spite of increasing light. Regression in such males is related either to changes within the animals, like fatigue or immunity reactions, or to an increase of lighting above the optimum or to both. It is possible that, in nature, the shortening days after June 21 hasten it or intensify its effects.

BIBLIOGRAPHY

Allanson, M., 1932. The Reproductive Processes of Certain Mammals. III. The Reproductive Cycle of the Male Ferret. *Proc. Roy Soc.*, Ser. B, **110**: 295.

Allanson, M., I. W. Rowlands, and A. S. Parkes, 1934. Induction of Fertility and Pregnancy in the Anoestrous Ferret. *Proc. Roy. Soc.*, Ser. B, **115**: 410.

Bachman, C., J. B. Collip, and H. Selye, 1934. Anti-Gonadotropic Substances. *Proc. Soc. Exper. Biol. Med.*, **32**: 544.

BISSONNETTE, T. H., 1932. Modification of Mammalian Sexual Cycles. Reactions of Ferrets (Putorius vulgaris) of Both Sexes to Electric Light Added after Dark in November and December. *Proc. Roy. Soc.*, Ser. B, **110**: 322.

BISSONNETTE, T. H., 1933. Light and Sexual Cycles in Starlings and Ferrets. *Quart. Rev. Biol.*, **8**: 201.

HILL, M., AND A. S. PARKES, 1933. Studies on the Hypophysectomized Ferret. IV. Comparison of the Reproductive Organs during Anoestrus and after Hypophysectomy. V. Effect of Hypophysectomy on the Response of the Female Ferret to Additional Illumination during Anoestrus. VI. Comparison of the Response to Oestrin of Anoestrous, Ovariectomized and Hypophysectomized Ferrets. *Proc. Roy. Soc.*, Ser. B, **113**: 530.

HILL, M., AND A. S. PARKES, 1934. Effect of Absence of Light on the Breeding Season of the Ferret. *Proc. Roy. Soc.*, Ser. B, **115**: 14.

MARSHALL, F. H. A., AND F. P. BOWDEN, 1934. The Effect of Irradiation with Different Wave-lengths on the Oestrous Cycle of the Ferret, with Remarks on the Factors Controlling Sexual Periodicity. *Jour. Exper. Biol.*, **11**: 409.

FURTHER ANALYSIS OF THE PROTECTIVE VALUE OF BIOLOGICALLY CONDITIONED FRESH WATER FOR THE MARINE TURBELLARIAN, PROCERODES WHEATLANDI. IV. THE EFFECT OF CALCIUM

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In a series of studies (1928, 1929, 1933) Allee has shown that the marine turbellarian, *Procerodes wheatlandi*, resists cytolysis longer in extremely hypotonic water, other conditions being equal, if such water has been biologically conditioned than if it lacks biological contamination. These relations hold even though the total concentration of electrolytes, as measured by the conductivity method, is the same. The balance of electrolytes is necessary in such experimental tests, since an increase in their concentration in the proportions found in sea water likewise confers protection.

Protective biological conditioning results when other *Procerodes* have previously been exposed to the fresh water, and especially so if some few *Procerodes* have recently died and disintegrated in it. The protective action of this biologically conditioned fresh water is maintained after dialysis to the same conductance as that of extremely dilute sea water controls. Water extracts of marine amphipods and of fresh water planarians, *Euplanaria novangliæ* (= *Planaria maculata*), show these same effects, as does water from cultures of the latter and water from sterilized hay infusions of a practically pure culture of *Paramecium*. The protective effects are not due to pH differences, or to a depressing action such as is produced by exposure to dilute alcohol or to ethyl urethane. The protective agent is not adsorbed by activated charcoal, or by coagulated egg white.

The onset of cytolysis was determined in these experiments by inspection with a hand lens. The results have been confirmed by histological studies on worms taken from Allee's 1933 experiments (Fowler, 1935).

Pantin (1931*a* and *b*) and Weil and Pantin (1931) have studied the adaptation of *Procerodes* (= *Gunda*) *ulvæ* to fresh waters. Their results bear on our problem since the two species are closely related taxonomically and in habitat toleration. *P. ulvæ* invades small streams

where conditions are suitable; it may extend up these to, or slightly above, mean high water at neap tides (Ritchie, 1934). The reaction of this planarian in fresh waters varies with the chemical composition of the water. The worms swell less and recover more completely on their return to sea water if placed in fresh waters rich in calcium rather than in calcium-deficient waters. In nature, Ritchie (1934) has found them living in fresh water with as little as 5 mgm. per liter of calcium for as long as five days when periods of calm weather, with the resulting lack of salt splash, coincide with neap tides.

Materials and Methods

P. wheatlandi were collected daily from the same locality as in the previous studies of Allee. *Euplanaria novangliæ* were collected from a fresh water pond known to be low in salt content; the pond water used came from the same pond which was also the source of these planarians and of pond water in Allee's latest work (1933).

The interval before the beginning of cytolysis in hypotonic waters depends on several factors, among which are the following: Small worms begin and undergo cytolysis more rapidly than do larger ones; worms long in the laboratory are less resistant than are those freshly collected; the more often the medium is renewed, the sooner cytolysis begins. The higher the temperature, or the lower the specific conductance of the water, the less the resistance of *Procerodes* (cf. Allee, 1933).

Assays of the protective value of various media were made, keeping these factors as nearly identical as possible for the worms whose resistance was being comparatively tested except that variations in specific conductance were used as a tool in the analyses. The worms were first isolated in a small drop of sea water on the curved bottom of the salt cellar type of watch glass; they were then washed five times with the respective media into which they were to be placed. The worms were paired for comparative assays before the test began; the members of each pair were selected for similarity in size, activity, and laboratory age. After the washings, 2 cc. of the respective media were run over each worm; this liquid was renewed every one, two, or four hours, depending on the severity of the media and the laboratory age of the worms. The survivals were all tested at room temperature in a room with north exposure which was not subjected to rapid changes in temperature. The highest temperature recorded for the summer in this room was 25.5° C. Examinations were made every fifteen minutes with a ten-power hand lens until the beginning of fatal cytolysis was noted. Ten worms were isolated into each type of media being tested in an assay.

Conductivity measurements were made using a No. 7651 Leeds and Northrup potentiometer and a Washburn type conductivity cell designed for solutions with low conductance. Specific conductances are given in mhos $\times 10^5$. Allee has previously given conductivity measurements in terms of ohms resistance. His data may be compared with those given in the present report by the use of the following data: under the conditions given, 6,000 ohms $= 5.42 \times 10^{-5}$ mhos; 2,500 ohms $= 13.05 \times 10^{-5}$ mhos and 1,700 ohms $= 19.20 \times 10^{-5}$ mhos.

Calcium analyses were made using the Van Slyke and Sendroy (1929) method. The accuracy of this method to within the one per cent claimed for it was confirmed by tests on the recovery of calcium added to distilled water and to the other media used in this work.

TABLE I

The Protective Action of Calcium for Procerodes Isolated into Extremely Hypotonic Water

Experiment No.	Medium	Calcium added		Calcium not added		Difference in hours
		Mhos $\times 10^5$	Hours survived	Hours survived	Mhos $\times 10^5$	
5	Tap	8.38	2.74	1.80	7.30	0.94
6	Sea	30.12	2.23	1.25	28.69	0.98
6*a*	Tap	8.83	3.95	2.43	7.15	1.52
8	Tap	21.29	9.33	4.00	7.07	5.33
8*a*	Sea	40.80	9.31	4.03	26.53	5.28
9	Pond	5.02	7.20	3.30	4.79	3.90
10	Pond	5.50	5.50	3.55	4.79	1.95
10*a*	Pond	6.27	15.80	5.32	4.56	10.48
11	Pond	7.43	3.23	2.14	4.79	1.09
12	Pond	7.13	2.63	2.20	4.79	0.43
13	Pond	5.40	4.34	3.00	4.79	1.34
21	Pond	6.25	5.98	4.08	4.79	1.90
Averages			6.02	3.09		2.92

Statistical probability 0.005

EXPERIMENTAL RESULTS

Calcium, as $CaCl_2$, was added to tap, pond and to extremely hypotonic sea water in a group of twelve experiments with results which are summarized in Table I. In each assay, the worms survived longer, on the average, in the water to which calcium had been added than did control worms in otherwise similar water. The amount of calcium used differed in different tests; in all cases the increase in specific conductance is a measure of the calcium added. Further comparisons can be made from the fact that in Experiment 8, 1 cc. of M/10 $CaCl_2$ was

added to 200 cc. of tap water and in Experiment 8*a* the same amount was added to 200 cc. of 1 : 300 sea water.

Although varying amounts of calcium were added and further variations were caused by differences in laboratory age and in the number of changes of the media during the assays, when the data given in Table I are considered as twelve paired experiments, the difference in survival of 2.92 hours has a statistical probability of 0.005.[1] The coefficient of correlation between the differences in the amount of calcium as measured by specific conductance and the differences in the resistance of the worms is 0.3691; this is statistically significant.

TABLE II

Relative survival of Procerodes isolated in tap water and in dilute sea water. Time is given in hours and each value is the average for 10 worms; specific conductance is in mhos.

Experiment No.	Dilute sea water		Tap water	
	Survival	Sp. cond. × 10^3	Survival	Sp. cond. × 10^3
1	4.825	19.07	5.725	6.64
2	6.667	20.14	8.566	6.38
3	4.697	25.90	7.865	6.79
4	2.768	28.69	2.100	7.82
5	1.535	27.95	1.800	7.30
6	1.250	28.69	2.425	7.15
7	7.875	39.75	8.150	7.07
8	4.775	26.53	4.000	7.07
Averages	4.299	27.09	5.079	7.03

The relatively greater protection from hypotonic water furnished by calcium in comparison with that given by other electrolytes in the proportions found in sea water is illustrated by the data summarized in Experiments 8 and 8*a*, Table I, and the further data given in Table II. In Experiments 8 and 8*a*, despite the differences in conductance, there is no significant difference in survival of worms in tap water or in 1 : 300 sea water and again there is no significant difference in survival in these two waters after the same amount of calcium is added. There is a decidedly significant difference between the survival of the worms in either water before and after the addition of calcium.

The waterworks of Falmouth, which supplies the tap water used in these experiments, now uses a lime treatment which materially increases the calcium content. This treatment was started since Allee's last pre-

[1] Values of 0.05 or less are usually considered statistically significant. "Student's" method of paired comparisons was used in all statistical analyses.

ceding experiments. The presence of this calcium probably accounts for the markedly greater protection of *Procerodes* in this water over that which would be expected from its low specific conductance. The dilutions of sea water contained approximately four times as much electrolytes as did the tap water; rough calculations of the calcium content from analyses furnished by the Falmouth waterworks showed this to be practically the same in the two. The survival of the *Procerodes* isolated in the two types of water was approximately the same in both;

TABLE III

Survival of Procerodes in planarian-conditioned fresh water as compared with that shown in different control media. Ten worms were assayed in each experiment except No. 13 in which eight were used.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
9	4.025	3.300	7.900	—
10	3.925	3.550	4.650	—
11	3.650	2.650	3.725	2.150
12	2.525	2.275	2.725	2.200
13	4.156	2.594	4.219	3.188
20	1.525	0.750	1.350	1.225
21	5.250	2.925	5.975	4.075
Means	3.579	2.578	4.363	2.568

Statistical analysis

	Difference		Probability	Cases
I–II	0.9900		0.0000	68
III– I	0.9375		0.0004	68
III–II	1.7941		0.0000	68
I–IV	0.8490		0.0010	48
IV–II	0.3125		0.2508	48
III–IV	1.0313		0.0000	48

the mean difference of 0.78 hours longer survival in tap water lacks statistical significance ($P. = 0.133$).

It is evident from these data that calcium delays the onset of cytolysis when *Procerodes* are isolated in extremely hypotonic fresh waters. Any factor which would tend to increase the amount of calcium present then, should confer such protection. This leads to the fundamental question of the present inquiry: Is the protective value of biologically conditioned water to be attributed to a differential increase in calcium

which accompanies conditioning? It is at once obvious that the addition of sea water to a control sample of fresh water until its total electrolyte content equals that of a biologically conditioned sample as measured by the conductivity method, while furnishing a basis for an adequate check on protection due to increased osmotic pressure, gives inadequate control over any specific electrolyte such as calcium. It is necessary to test directly for the amount of calcium present and to confirm its effectiveness in biologically conditioned water.

Unless otherwise stated, the amount of biological conditioning used was that furnished by 200 *Euplanaria novanglicæ* living in one liter of fresh pond water for approximately 44 hours, or its equivalent obtained by using fewer worms for a longer period. Sea water and calcium chloride were added respectively to other portions of fresh pond water until the specific conductance of all three solutions was the same. As an additional control, except in Experiments 9 and 10, a sample of untreated pond water was assayed together with the above media. The average survival times of *Procerodes* from seven such experiments are shown in Table III.

TABLE IV

Calcium analyses of assayed samples of media from Table III; results shown in mgm. calcium per liter.

	I Planarian-conditioned water	II Pond water	III Pond water + $CaCl_2$	IV Pond water + sea water
Calcium content.....	2.072	0.809	3.410	1.018

These data, together with the statistical analyses, show that planarian-conditioned water has a protective value lying between that of pond water plus sea water and pond water plus calcium chloride when all are of the same electrolytic content. When, however, the amount of calcium present in the planarian culture water (Table IV) was determined and calcium chloride was added to pond water to bring the calcium content to that of the planarian-conditioned water, no difference could be detected between the effectiveness of these two waters in protecting the *Procerodes* isolated in them. These results are summarized, together with a statistical analysis, in Table V.

To determine whether any factor other than calcium might be involved in the protection furnished by planarian-conditioning, a medium

TABLE V

Procerodes survival in pond water and planarian-conditioned pond water with the same calcium content.

Experiment No.	Hours of survival			
	I Planarian-conditioned water	II Pond water	III Pond water + sea water	IV Pond water + $CaCl_2$
26	5.250	5.650	3.850	8.300
27	1.875	2.150	1.150	3.500
Means	3.563	3.900	2.500	5.900

Statistical analysis

	Difference	Probability	Cases
I– II	−0.3375	1.0000	20
I–III	1.0625	0.0396	20
IV– I	2.3375	0.0052	20
II–III	1.4000	0.0230	20
IV– II	2.0000	0.0344	20
IV–III	3.4000	0.0000	20

was made up to represent a synthetic river water (Henderson, 1913, p. 113). To each liter of water the following salts were added:

100 mgm. $CaCl_2$
50 mgm. $MgSO_4$
25 mgm. NaCl
10 mgm. KCl

This water in full, half- and quarter-strengths, when conditioned by planarians, showed no change in the total electrolytic content; likewise there was no protection for *Procerodes* when compared by the usual assay to the survival shown in similarly treated but unconditioned synthetic river water. The results of these experiments together with a statistical analysis are summarized in Table VI. From all this evidence, we can safely conclude that an increase in calcium is the factor furnishing the protection to *Procerodes* in planarian-conditioned pond water.

Allee's previous work had shown that water extracts of *Procerodes* themselves furnished a potent protection for other worms of the same species isolated in extremely hypotonic media. The relation between this protection and the calcium content of the water was also tested.

TABLE VI

A summary of tests which indicate that the protection furnished by calcium is sufficient to account for the protective value of planarian-conditioned water. Each of the survival times given is the average for ten worms.

Experiment number	Hours survival		Amount of conditioning	Laboratory age of *Procerodes*	Mhos × 10^6	Media strength	Media changed	Difference
	Planaria culture	Control						
14	10.225	5.625	100 worms in 250 cc. 16 hrs.	1 day	48.70	Full	Every hour	+4.60
15	6.550	6.800	100 worms in 500 cc. 44 hrs.	2 days	48.70	Full	Every hour	−0.25
16	21.75	19.30	14 worms in 250 cc. 68 hrs.	over 5 days	48.70	Full	Every hour*	+2.45
17	5.65	6.30	200 worms in 500 cc. 20 hrs.	3 days	26.15	Half	Every 2 hours	−0.65
18	6.95	7.15	100 worms in 500 cc. 44 hrs.	4 days	26.15	Half	Every 2 hours	−0.20
19	7.00	6.20	60 worms in 500 cc. 68 hrs.	4 days	26.15	Half	Every 2 hours	+0.80
23	5.50	6.90	200 worms in 500 cc. 20 hrs.	1 day	12.32	Quarter	Every 2 hours	−1.90
24	8.90	9.35	100 worms in 500 cc. 44 hrs.	2 days	12.32	Quarter	Every 2 hours	−0.45
			Average					+0.55
			Statistical Probability					0.4500

* Media exhausted after six changes.

Procerodes extract was prepared as in the earlier work. After five washings with distilled water, 500 *Procerodes* were boiled in distilled water for about 15 minutes. The beaker was then covered and set aside for 24 hours. Controls were made up to the same specific conductance as the extract by adding calcium chloride to distilled water and sea water to distilled water respectively. All three media were then assayed for their protective value to *Procerodes* and were then analyzed for calcium. It is evident from an examination of Tables VII and VIII which summarize these data that calcium is also the factor in *Procerodes* extracts

TABLE VII

The protective value of *Procerodes* extracts for *Procerodes* isolated in hypotonic media; each survival time given is the average for ten worms.

Experiment No.	Hours survival		
	I *Procerodes* extract	II Dist. H_2O + $CaCl_2$	III Dist. H_2O + sea water
28	6.150	6.350	4.150
29	2.100	3.000	1.750
Means	4.125	4.675	2.950
	Statistical analysis		
	Difference	Probability	Cases
I– II	0.550	0.5280	20
I–III	1.175	0.0344	20
II–III	1.725	0.0050	20

TABLE VIII

Calcium analyses of assayed samples from Table VII; results shown in mgm. per liter.

	Procerodes extract	Dist. H_2O + $CaCl_2$	Dist. H_2O + sea water
Calcium content	7.20	8.66	0.42

which furnishes the observed protection for *Procerodes*. Since making a water extract of *Procerodes* is a short-cut method for preparing *Procerodes*-conditioned water, it is unnecessary to examine this possibility for further confirmatory evidence that an increase in calcium is the fac-

tor furnishing protection to *Procerodes* in biologically conditioned fresh waters.

Discussion

These experiments were carried on by essentially the same methods used by Allee in his preceding studies and support his results wherever similar points were being tested. Specifically, in addition to more general relations, these experiments confirm the earlier findings, that, other conditions being equal, *Procerodes* survive longer (1) if the osmotic pressure is increased by the addition of sea water; (2) if the hypotonic water is biologically conditioned (*a*) by the presence of living fresh water planarians or (*b*) by the presence of water extracts of freshly killed *Procerodes*.

In addition, the earlier results are extended by the demonstration that there is more calcium added than would be expected from its proportionate concentration in sea water and that calcium has protective value greater than would be expected from its osmotic effect. There is nothing in the earlier experiments which is out of harmony with the present findings.

In certain of Allee's work (1929, 1933) the conditioned water was dialyzed to bring the specific conductance to the desired experimental level. Such dialyzed solutions were definitely protective. This protection can be explained adequately in the light of the present work if one considers the amounts of calcium introduced into conditioned water, for example, by *Procerodes* extracts (cf. Table VIII). Even if one assumes that the collodion membranes used were freely permeable to calcium, the calcium content could be reduced by more extreme dialysis than that used and still leave enough calcium to be definitely protective as compared with a similarly hypotonic sea water control.

The demonstration that the protective action of these biologically conditioned solutions is due to the increased calcium content in so far as the resistance of *Procerodes wheatlandi* to hypotonic water is concerned, brings this phenomenon into line with the greater resistance shown by *Procerodes* (= *Gunda*) *ulvæ* to fresh water when the calcium content is high. This is hardly the place, nor is *Procerodes* necessarily the most favorable material for an inquiry into the mechanism of calcium protection under these conditions.

The analyses of Pantin and his associates and of Beadle (1931, 1934) indicate that *P. ulvæ* lives in osmotic equilibrium with sea water and reaches a steady physiological state in certain fresh waters. This steady state is not the result of simple osmotic balance but is dependent

largely on the calcium content of the water. It is maintained in part by a change in the permeability of the surface epithelium and is accompanied by increased respiration; it cannot be maintained indefinitely in extremely hypotonic waters. It seems highly probable (cf. Weil and Pantin, 1931) that the mechanism with these planarians is related to that in other cells in certain of which it has been shown, *Arbacia* eggs for example (McCutcheon and Lucke, 1928), that calcium decreases cell permeability to water and (Heilbrunn, 1930) favors membrane formation at a broken surface. The protection in these cases appears to be due to a decreased water intake rather than to a decreased leaching out of materials essential for continued existence.

We have not investigated the range of concentrations of calcium which would delay cytolysis in *Procerodes* placed in hypotonic water; in fact, we have established neither the upper nor the lower effective concentration. We know that an increase in specific conductance produced by the addition of calcium chloride equal to 1.08×10^{-5} mhos will give measurable protection. A rough calculation shows that this is approximately equivalent to the introduction of M/2600 $CaCl_2$. This approaches the lower limit of effectiveness under the conditions of our experiments. Buchanan (1935) reports that complete cytolysis readily occurs in *Euplanaria dorotocephala* in distilled water while in distilled water solutions of $CaCl_2$ there is no cytolysis in concentrations from M/500 to M/40,000, and cytolysis is distinctly delayed in a dilution of M/100,000. He found a detectable protective action in a M/1,000,000 solution.

We have been engaged in investigating the relation between calcium and the protective action of biological conditioning in extremely hypotonic waters when the calcium content is relatively low. We have little evidence regarding the effectiveness or non-effectiveness of such conditioning when the calcium is high. There are some indications in the first three significant lines in Table VI where full strength synthetic river water was used, that such conditioning may be effective. If so, some other mechanism than an increase in calcium is probably acting. These conditions approximate those found when a hard water stream from a limestone region flows into the ocean (cf. Breder, 1934). There is evolutionary and ecological as well as physiological interest in the relations between numbers present and the effectiveness of the invasion of such waters. Our present work does not deal with this question. However, it does show that marine invasions by animals with the physiological requirements of *Procerodes* into such relatively soft fresh waters as we have investigated is definitely favored by the presence in these waters of calcium excretors such as *Euplanaria novangliæ* has been shown to be.

We have no information concerning the actual importance of such conditioning under natural conditions.

Throughout these studies there has been evidence of two opposing effects of numbers upon the resistance of these worms to fresh water; (*a*) the protective effect of freshly conditioned water or of numbers of animals present and (*b*) the harmful effect of numbers which shows up in conditioned media that have been allowed to go stale. At the osmotic level of these experiments, the protective effect is due to an increase in calcium and while the harmful effect has not been analyzed, it is a good *a priori* guess that the observed ill effects are associated with the accumulation of waste products of metabolism or to decomposition products of these.

The series of experiments of which this is the fourth report, were begun and have been prosecuted primarily in an investigation of a phase of mass physiology associated with animal aggregations (cf. Allee, 1931, 1934). The original impetus towards these particular experiments came from the observations of Drzewina and Bohn (1920, 1928) that the marine turbellarian *Convoluta roscoffensis* survives longer in hypotonic water if present in numbers than if isolated. This protection they attributed to the more rapid production of some sort of autoprotective substance by the group than would be possible for a single individual. This suggestion is now seen to have been essentially correct and, under the conditions of our experiments, to be composed of nothing more mysterious than calcium.

Summary

1. An increase in the calcium content of extremely hypotonic water, when the calcium content is less than that found in sea water, delays the onset of fatal cytolysis for *Procerodes wheatlandi* isolated in such media.

2. Other electrolytes, even when increased appreciably, do not show this same protection; however, some protection of an osmotic nature is apparent.

3. The increase of calcium in biologically conditioned fresh waters is adequate to explain their observed protection for *Procerodes*.

LITERATURE CITATIONS

Allee, W. C., 1928. Studies in Animal Aggregations: Mass protection from fresh water for Procerodes, a marine turbellarian. *Jour. Exper. Zool.*, **50**: 295.

Allee, W. C., 1929. Studies in Animal Aggregations: Mass protection from hypotonic sea-water for Procerodes, a marine turbellarian, with total electrolytes controlled. *Jour. Exper. Zool.*, **54**: 349.

Allee, W. C., 1931. Animal Aggregations: A Study in General Sociology. University of Chicago Press.

ALLEE, W. C., 1933. Studies in Animal Aggregations: Further analysis of the protective value of biologically conditioned fresh water for the marine turbellarian, Procerodes. *Physiol. Zool.*, **6**: 1.

ALLEE, W. C., 1934. Recent Studies in Mass Physiology. *Biol. Rev.*, **9**: 1.

BEADLE, L. C., 1931. The Effect of Salinity Changes on the Water Content and Respiration of Marine Invertebrates. *Jour. Exper. Biol.*, **8**: 211.

BEADLE, L. C., 1934. Osmotic Regulation in Gunda ulvæ. *Jour. Exper. Biol.*, **11**: 382.

BOHN, G., AND A. DRZEWINA, 1920. Variations de la sensibilité à l'eau douce des Convoluta, suivant les états physiologiques et le nombre des animaux en expérience. *Compt. Rend. Acad. Sci.*, **171**: 1023.

BREDER, C. M., JR., 1934. Ecology of an Oceanic Fresh-Water Lake, Andros Island, Bahamas, with Special Reference to its Fishes. *Zoologica*, **18**: 57.

BUCHANAN, J. W., 1935. An Analysis of 'Physiological States Responsible for Antero-posterior Disintegration in Planaria dorotocephala. *Biol. Gen.*, in press.

DRZEWINA, A., AND G. BOHN, 1928. Les Convoluta. *Ann. Sci. Nat. Zool., X.*, Ser. **11**: 299.

FISHER, R. A., 1925. Expansion of "Students'" Integral in Powers of N^{-1}. *Metron*, **5**: 109.

FOWLER, LILLIAN HENDERSON, 1935. Relative Effect of Biologically Conditioned and Other Extremely Hypotonic Water on the Tissues of the Marine Turbellarian, Procerodes wheatlandi. *Physiol. Zool.*, **8**: No. 2.

HEILBRUNN, L. V., 1930. The Action of Various Salts on the First Stage of the Surface Precipitation Reaction in Arbacia Egg Protoplasm. *Protoplasma*, **11**: 558.

HENDERSON, L. J., 1913. The Fitness of the Environment. Macmillan.

MCCUTCHEON, M., AND B. LUCKÉ, 1928. The Effect of Certain Electrolytes and Non-electrolytes on Permeability of Living Cells to Water. *Jour. Gen. Physiol.*, **12**: 129.

PANTIN, C. F. A., 1931*a*. The Adaptation of Gunda ulvæ to Salinity: I. The environment. *Jour. Exper. Biol.*, **8**: 63.

PANTIN, C. F. A., 1931*b*. The Adaptation of Gunda ulva to Salinity: III. The electrolytic exchange. *Jour. Exper. Biol.*, **8**: 82.

RITCHIE, A. D., 1934. Habitat of Procerodes ulvæ. *Jour. Mar. Biol. Ass.*, **19**: 663.

"STUDENT," 1925. New Tables for Testing the Significance of Observations. *Metron*, **5**: 105.

VAN SLYKE, D. D., AND J. SENDROY, JR., 1929. Gasometric Determination of Oxalic Acid and Calcium, and its Application to Serum Analysis. *Jour. Biol. Chem.*, **84**: 217.

WEIL, E., AND C. F. A. PANTIN, 1931. The Adaptation of Gunda ulvæ to salinity. II. The water exchange. *Jour. Exper. Biol.*, **8**: 73.

ON THE INFLUENCE OF PYOCYANINE ON THE RESPIRATION OF THE SEA URCHIN EGG

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Barron (1929) has demonstrated that the respiration of the unfertilized *Arbacia* and *Asterias* egg is increased by methylene blue. Runnström (1930) reported similar observations for the *Paracentrotus* egg. Runnström (1930, 1932) and Örström (1932) studied chiefly the influence of dimethylparaphenylene diamine on *Paracentrotus eggs.* This compound penetrates the eggs and causes a considerable increase of respiration both of the fertilized and the unfertilized eggs. The respiration of the eggs in the diamine solution is inhibited by cyanide and carbon monoxide. The degree of inhibition by cyanide is the same in the unfertilized and fertilized diamine eggs. The same holds true for the inhibition by CO, if the respiration is equal in the unfertilized and fertilized diamine eggs. The degree of inhibition by CO is dependent on the rate of reduction of the iron-containing enzyme of Warburg (1932). The higher the rate of reduction the more accessible is the enzyme to CO, which reacts with the reduced form of the enzyme (Warburg). Runnström inferred from his inhibition experiments that dimethylparaphenylene diamine is not auto-oxidized in the sea urchin egg but must be oxidized by the reduction of the iron-containing enzyme. Further, it was inferred that this enzyme is also present in the unfertilized egg in a reactive state, but the rate of oxidation is limited by a block in the chain of "carriers" (Keilin, 1929) which connects the iron-containing enzyme and the substrate-dehydrase system.

Barron did not find any inhibition of the respiration enhanced by methylene blue on addition of cyanide. This increase of the respiration is not due to a higher activity of the iron-containing enzyme. Methylene blue is reduced by systems in the cell which cannot react directly with molecular oxygen. It seemed to the writer of interest to try the action of pyocyanine on the respiration of the sea urchin egg. Pyocyanine belongs with respect to its oxidation reduction potential to the same range as methylene blue. As shown by Friedheim and Michaelis (1931) and further by Michaelis, Hill, and Schubert (1932),

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the pyocyanine can be oxidized or reduced in two steps, each step involving the transfer of one electron. Methylene blue, on the other hand, is oxidized or reduced in one step, each step involving the transfer of two electrons. This difference may be physiologically significant. Friedheim (1931) has shown that pyocyanine is a more effective catalyzer of some oxidations than methylene blue. Further (Friedheim, 1934), he found that the aerobic glycolysis of tumors can be decreased in presence of pyocyanine. The present writer and Michaelis (Runnström and Michaelis, 1934) have proved that the action of methylene blue and pyocyanine is different in the system hemolyzed red blood cells plus hexosephosphate. The oxygen uptake is the same with both dyestuffs, but in the presence of pyocyanine a coupling between the respiration and the synthesis of phosphate esters is induced, which does not exist or is much less conspicuous in the same system containing methylene blue.

TABLE I

Unfertilized Eggs	Control	Pyocyanine			
		0.006%	0.009%	0.012%	0.015%
cu. mm. O_2 in 150 min.....	30	75	84.5	93	93
Increase, *per cent*		125	180	210	210
Fertilized Eggs					
cu. mm. O_2 in 150 min.....	112	128	204	212	220
Increase, *per cent*		14	80	90	96

Suspension: 4.5 per cent.

The oxygen consumption was measured in the present research by Warburg's manometric method. The type of vessels used was that described by Borei (1934). It has proved to be useful for experiments with the sea urchin eggs. Into each vessel always 3 cu. cm. of the egg suspension was introduced. A 5 per cent solution of KOH was introduced into the inset as well as into one of the two side arms. The concentration of the suspension was determined by centrifuging in capillary tubes as described by Runnström (1933). The values obtained were corrected for the interstitial water by multiplication with the factor 0.64, cf. Teissier (1929). All conclusions are based on comparisons between values obtained in experiments with uniform material. Thus the exact absolute concentration of the suspensions is of minor importance.

Table I gives the evidence that the respiration is increased considerably in both fertilized and unfertilized eggs on addition of pyocyanine. The percentage increase is much higher in the unfertilized than in the

fertilized eggs. On the other hand, the absolute values of the oxygen consumption in the unfertilized eggs are lower than those of the fertilized eggs. The highest value obtained in the unfertilized eggs is still somewhat below that found in the normal fertilized eggs. The eggs can be fertilized in the solution of pyocyanine in sea water. As can be predicted from Table I, the percentage increase of the oxygen consumption is much lower than under normal conditions. At the concentration 0.006 per cent pyocyanine the increase is only 75–125 per cent, while in the control the increase amounts to 400–500 per cent. The eggs treated with pyocyanine were transferred at the end of the measurements to normal sea water. The unfertilized eggs are in no way activated and could be fertilized. The division of the fertilized eggs took place in the pyocyanine solution. In the higher concentrations (0.012–0.015 per cent) the division was, however, delayed or suppressed in spite of the increased respiration. The eggs developed after transfer to normal sea water to the pluteus stage. A certain injurious effect of

TABLE II

	Unfertilized Eggs					
Pyocyanine, *per cent*	0	0.003	0.006	0.02	0.02	0.02
HCN n	—	—	—	—	0.001	0.0025
cu. mm. O_2 in 120 min.	24	36	64	82	110	128
Increase, *per cent*	—	49	167	240	360	435

the higher concentrations of pyocyanine was evident, even after transfer to normal sea water.

An *increase* of the oxygen consumption was regularly observed on addition of HCN to the unfertilized eggs in pyocyanine.

In the highest HCN concentration the oxygen consumption was equal to or somewhat higher than in the fertilized control eggs.

The same material was used for the experiments on which Tables II and III are based. The figures are thus directly comparable. The respiration of the fertilized pyocyanine eggs is decreased by addition of HCN. The residual respiration is about 70 per cent. From other experiments the residual respiration in presence of 0.0025 N HCN is known to be only about 30–35 per cent. It is easy to see that the part of the respiration induced by pyocyanine, $266 - 123 = 140$ cu. mm., is not inhibited by HCN: 30 per cent of 123 is about 40 cu. mm.; $140 + 40$ cu. mm. is equal to 180 cu. mm., which is the oxygen consumption observed in the eggs in pyocyanine plus HCN. In some

cases the residual respiration was somewhat higher than expected according to the above calculation but there is always an inhibition.

No data are available about the HCN inhibition in the unfertilized eggs of *Arbacia* in sea water without pyocyanine, but to judge from figures (Runnström, 1930) valid for the *Paracentrotus* egg, the inhibition is weak in the normal (not aged) unfertilized egg as compared with the inhibition in the fertilized egg.

The rise of the oxygen consumption of the eggs in sea water plus pyocyanine is greater than on addition of methylene blue. In one experiment the increase of respiration in unfertilized eggs was 60 per cent in presence of 0.006 per cent methylene blue, while the increase caused by 0.006 per cent pyocyanine (a concentration very nearly equimolecular to that of the methylene blue in this experiment) was 200 per cent.

At the suggestion of Dr. Alfred Mirsky, the eggs were frozen by means of solid CO_2 and ether at a temperature of about —80° C. These

TABLE III

	Fertilized Eggs		
Pyocyanine, *per cent*	0	0.02	0.02
HCN n	—	—	0.0025
cu. mm. O_2 in 120 min	123	266	181

Suspension: 4 per cent.

eggs were then allowed to thaw slowly at room temperature. The thawing breaks up the eggs. A part of the egg contents is dissolved in the sea water, another part remains undissolved. The thawed eggs show a certain oxygen uptake, but this is considerably enhanced by the addition of pyocyanine or methylene blue. In many experiments hexose phosphate was added, which increased the oxygen uptake 100–200 per cent. (Table IV.)

Pyocyanine gives a somewhat higher oxygen consumption than methylene blue added to the above system with hexose phosphate. In one experiment the oxygen consumption in presence of methylene blue was 97 cu. mm., while in presence of pyocyanine it was 123 cu. mm. in 120 minutes.

In the experiments cited above (Table III) it is evident that the respiration in fertilized eggs to which HCN has been added is increased by addition of pyocyanine to a level above the normal. The concentration of HCN in question blocks the mitotic process. If the eggs were brought into the HCN sea water soon after fertilization, the

division stopped in an early prophase, in the stage characterized in the living material by the appearance of the clear streak near to the nucleus. A certain increase of the volume of the nucleus takes place but the dissolution of the nuclear membrane does not occur in the presence of HCN. The block is due without any doubt to the depression of the respiration by HCN. The effect is the same in nitrogen containing traces of oxygen. In pure nitrogen even the fusion of the pronuclei and the swelling of the sperm nucleus is prevented. The normal level of oxygen consumption is more than restored by the addition of pyocyanine to the egg suspension with HCN, *yet the division is stopped at*

TABLE IV

Unfertilized eggs remained frozen during four days. After thawing the suspension was diluted 70 per cent with distilled water.[2]

Hexose monophosphate m.	—	0.012
Pyocyanine, %	0.006	0.006
cu. mm. O_2 in 130 min.	55	140

the same stage as without pyocyanine. The increased respiration is quite ineffective, when caused by the presence of pyocyanine. The eggs were also kept in pure nitrogen and it was found that the division is completely inhibited even in presence of pyocyanine, the concentration of which was varied from 0.006–0.02 per cent. The eggs were brought almost immediately after fertilization into Erlenmeyer flasks closed by rubber stoppers which were pierced by two glass tubes. Nitrogen, purified over heated reduced copper, was bubbled through the flasks. After two hours the bubbling of nitrogen was stopped and the stoppers removed. It was found that gradually the development started again. The rate of recovery is the same with and without the pyocyanine. Our results agree with those of Örström (cf., Runnström, 1933) according to which methylene blue does not promote the development of the *Paracentrotus* egg under anaerobic conditions. It seems that the opposite results reported by Rapkine (1929) are due to some technical error. The cell division can be completed only if the eggs are subject to anaerobic conditions after the dissolution of the nuclear membrane. Even then the block sets in again as soon as the segmentation furrow is formed. The respiration is necessary for the division of the sea urchin egg. This must mean that the respiration is coupled to processes in the cell necessary for the division. It is most likely that the reversible oxidation reduction systems acting as "carriers" (Keilin) bring about the coupling between the respiration and other processes in the cell. In

[2] The undiluted suspension shows a somewhat lower oxygen uptake, probably a salt effect.

the system hemolyzed blood cells plus hexose phosphate referred to above, the pyocyanine can act as a "gear" between the respiration and the synthesis of phosphate compounds. From the reported results it must be inferred, however, that in the more complicated system, the fertilized egg cell, pyocyanine cannot act as a gear between the respiration and the processes connected with the completion of the cell division.

It is quite evident from the above data that pyocyanine is auto-oxidized in the sea urchin egg. In this respect it differs from dimethyl-paraphenylene diamine but resembles methylene blue according to the experiments carried out by Barron. Friedheim (1931) reports, however, that the respiration of *Bacillus pyocyaneus* enhanced by addition of pyocyanine is decreased by CO as well as by HCN. This indicates that the pyocyanine in this case does not react or reacts slowly with the molecular oxygen. It must be oxidized by the iron-containing enzyme. This difference as compared with the conditions in the sea urchin egg may perhaps be due to differences of pH in the interior of the cells in question.

In the unfertilized egg no part of the respiration is inhibited by HCN in presence of pyocyanine. On the contrary, the respiration is considerably enhanced by the presence of HCN. This fact cannot be explained at present but it may be remembered that HCN possibly can activate certain enzymes, the action of which increase the amount of substrate present.

In the fertilized egg the part of the respiration due to the addition of pyocyanine is not inhibited by HCN. In the fertilized egg with pyocyanine there are two auto-oxidizable systems, the iron-containing enzyme and pyocyanine. This may account for the higher absolute level of respiration observed in the fertilized eggs as compared with the unfertilized ones. In the non-aged unfertilized eggs the respiration is low. The action of the iron-containing enzyme is, according to the writer's assumption, checked by a block in the chain of "carriers." Possibly a minor part of the molecules of the iron-containing enzyme is reduced (Runnström, 1930). The possibility may also be considered that some more slowly auto-oxidizable system (like Warburg's "yellow enzyme" substrate-dehydrase) may account partly or wholly for the comparatively slow oxidations in the unfertilized eggs. Anyhow, on the addition of pyocyanine to the unfertilized eggs, the chain of oxidations apparently goes wholly over pyocyanine and not or to quite an insignificant degree over the iron-containing enzyme.

The results reported above clearly show that the dehydrase-substrate

system cannot be a limiting factor for the rate of respiration either in the fertilized or in the unfertilized egg.[8]

The experiments with dimethylparaphenylene diamine referred to above demonstrate, on the other hand, that the iron-containing enzyme is not the limiting factor. This points again to the "carriers" as the part of the respiratory mechanism which has to be made responsible for the considerable change in oxygen consumption following the activation of the egg.

This paper forms part of the work carried out by the writer in collaboration with Dr. L. Michaelis, and I wish to express my thanks to him for his generous help and valuable suggestions.

Summary

Addition of pyocyanine to a suspension of sea urchin eggs causes an increase of the respiration of the eggs. The percentage increase is higher in the unfertilized than in the fertilized eggs. The absolute values of the oxygen consumption in presence of pyocyanine are higher after than before fertilization. The respiration of the unfertilized eggs is enhanced in the presence of pyocyanine by the addition of HCN. In the fertilized eggs, on the contrary, the respiration enhanced by pyocyanine is always decreased by HCN. Pyocyanine is auto-oxidized in the sea urchin egg; the iron-containing enzyme is not involved in the oxidations induced by pyocyanine. The rate of oxidation induced by pyocyanine is somewhat higher than that of the respiration induced by methylene blue both in intact eggs and in eggs broken up by freezing and thawing. The block of division of the sea urchin egg caused by HCN or by anærobic conditions is not removed by addition of pyocyanine. In the fertilized eggs the respiration in the presence of pyocyanine and HCN can be higher than in the control; in spite of this the division stops at the same stage as when the respiration is decreased 75 per cent by

[8] Only the conditions prevailing in the egg immediately following the fertilization are considered here. During the ensuing development there is a gradual increase of respiration as found by Warburg (1926). The "increasing fraction" of the respiration is inhibited by lithium according to Lindahl (1933, 1934). The action of lithium on the increasing fraction of respiration can be accounted for by the law of mass action. Lithium possibly inhibits the formation of certain break-down products of carbohydrates by reacting with an enzyme or some part of an enzyme system. If this is true, the substrate-dehydrase system must gradually acquire the character of a limiting factor for the oxidation rate during the course of development. It may also be mentioned here that no increase of respiration was observed in living cells of baker's yeast on addition of pyocyanine or methylene blue. This holds true even if the respiration is inhibited by HCN. (Experiments carried out by M. Schüldt.) The situation in baker's yeast is very different from that in the sea urchin egg.

the addition of HCN alone. The general conclusion is drawn that neither the iron-containing enzyme nor the dehydrase-substrate system act as limiting factors for the oxidation rate in the unfertilized or newly fertilized egg.

LITERATURE

BARRON, E. S. G., 1929. *Jour. Biol. Chem.*, **81**: 445.
BOREI, H., 1934. *Zeitschr. vergl. Physiol.*, **20**: 380.
FRIEDHEIM, E. A. H., 1931. *Jour. Exper. Med.*, **54**: 207.
FRIEDHEIM, E. A. H., 1934. *Biochem. Jour.* **28**: 173.
FRIEDHEIM, E., AND L. MICHAELIS, 1931. *Jour. Biol. Chem.*, **91**: 355.
KEILIN, D., 1929. *Proc. Roy. Soc.*, Ser. B., **104**: 206.
LINDAHL, P. E., 1933. *Arch. entw. mech.*, **128**: 661.
LINDAHL, P. E., 1934. *Naturwiss.*, **22**: 105.
MICHAELIS, L., E. S. HILL, AND M. P. SCHUBERT, 1932. *Biochem. Zeitschr.*, **255**: 66.
ÖRSTRÖM, Å., 1932. *Protoplasma*, **15**: 566.
RAPKINE, L., 1929. *Compt. rend Acad. Sci.*, **188**: 650.
RUNNSTRÖM, J., 1930. *Protoplasma*, **10**: 106.
RUNNSTRÖM, J., 1932. *Protoplasma*, **15**: 532.
RUNNSTRÖM, J., 1933. *Protoplasma*, **20**: 1.
RUNNSTRÖM, J., AND L. MICHAELIS, 1934. *Science*, **80**: 167. (Full report in press.)
TEISSIER, G., 1929. *Arch. de zool. expér. et gén.*, **69**: 137.
WARBURG, O., 1926. Stoffwechsel der Tumoren. Berlin.
WARBURG, O., 1932. *Zeitschr. angewandte Chemie*, **45**: 1. (Review.)
WARBURG, O., AND W. CHRISTIAN, 1932. *Biochem. Zeitschr.*, **254**: 438.